Applied and Environmental Microbiology, July 2008, p. 4036–4043 Vol. 74, No. 13
0099-2240/08/$08.00+0 doi:10.1128/AEM.02609-07

Assessment of Gut Bacteria for a Paratransgenic Approach To Control *Dermolepida albohirtum* Larvae[▽]

Geoffrey W. Pittman,[1] Stevens M. Brumbley,[3] Peter G. Allsopp,[2] and Scott L. O'Neill[1]*

School of Integrative Biology, The University of Queensland, St. Lucia, Queensland 4072, Australia[1]; BSES Limited, P.O. Box 86, Indooroopilly, Queensland 4068, Australia[2]; and Australian Institute for Bioengineering and Nanotechnology, Indooroopilly, Queensland 4068, Australia[3]

Received 19 November 2007/Accepted 26 April 2008

Bacteria from the hindguts of *Dermolepida albohirtum* larvae were assessed for their potential to be used in paratransgenic strategies that target scarab pests of sugarcane. Bacteria isolated in pure culture from the hindguts of *D. albohirtum* larvae were from the *Proteobacteria*, *Firmicutes*, and *Actinobacteria* phyla and matched closely with taxa from intestinal and rhizosphere environments. However, these isolates were not the most common gut-associated bacteria identified in denaturing gradient gel electrophoresis (DGGE) hindgut profiles. Subsequently, eight species of gut bacteria were fed to larvae, and RNA-based DGGE analysis of 16S rRNA was used to detect the persistence of these isolates in the hindgut environment. One of these isolates (Da-11) remained metabolically active in the hindgut for 19 days postconsumption. Da-11 most likely forms a new genus within the *Burkholderiales* order, along with taxa independently identified from larvae of the European scarab pest, *Melolontha melolontha*. Using the EZ::Tn*5* transposon system, a kanamycin resistance gene was inserted into the chromosome of Da-11, thus establishing a stable transformation technique for this species. A second feeding trial that included inoculating approximately 400 transgenic Da-11 cells onto a food source resulted in a density of 1×10^6 transgenic Da-11 cells/ml in the hindguts of larvae at 9 days postconsumption. These populations were maintained in the hindgut for at least another 12 days. The successful isolation, genetic transformation, and establishment of transgenic Da-11 cells in the hindguts of *D. albohirtum* larvae fulfill fundamental requirements for the future development of a paratransgenic approach to control scarab pests of sugarcane.

Insect pests are well known for their negative impacts on human health and agriculture. The genetic modification of pest insects holds much promise as a future control method to reduce these impacts (35). However, the genetic modification of insects can be technically challenging in comparison to that of bacteria. An alternative approach, aimed at controlling insect pests and the pathogens they transmit, utilizes bacteria that are closely associated with the host insect (3, 14). In this paratransgenic approach, bacteria that are highly adapted to a particular pest insect are genetically transformed to express compounds that can either block the transmission of pathogens (14, 17) or directly harm the host insect (9, 39).

The selection of host-specific species can be critical in paratransgenic approaches that use gut bacteria to deliver transgenic compounds into the gut environment of pest insects. Indigenous gut bacteria are favored as paratransgenic delivery vehicles because nonresident bacteria are often unable to become established within a particular intestinal environment (9, 22, 23, 40, 43). Moreover, utilizing transgenic bacteria that are tightly associated with a single host species may reduce the impacts on nontarget insects. Additionally, the negative fitness effects often seen when transgenic organisms are released into competitive environments (24, 42) can potentially be reduced by the use of genes that assist in the establishment of transgenic strains in complex microbial environments (18).

In the hindguts of scarab larvae, a dense and diverse microbial community assists in the degradation of plant matter (8, 28). Members of the hindgut community that are associated with larvae of the Australian scarab pest, *Dermolepida albohirtum* (32), and the European scarab pest, *Melolontha melolontha* (15), are consistently found across the geographical distribution of the larval host. These commonly found gut bacteria are possible candidates in a biotechnological approach to control the feeding damage caused by scarab larvae in agricultural crops (32). This could be achieved by genetically engineering gut bacteria to express antifeeding compounds that target larvae and applying them to soil containing the pest.

The root-feeding activities of scarab larvae cause significant damage to many types of crops, including sugarcane. Soilborne pathogens have been used effectively as biocontrol agents against scarabs (25). However, in many cases, the commercial development of natural pathogens is challenging because they are often difficult and expensive to culture. Consequently, the control of scarabs in Australian sugarcane is limited to a reliance on chemical pesticides (34).

This paper reports the isolation of bacteria residing in the hindguts of larvae of *D. albohirtum* and an assessment of their ability to recolonize the hindgut environment. This process led to the discovery of a novel gut bacterium (Da-11) that most likely forms a new genus in the order *Burkholderiales*, along with taxa independently identified from the larval hindgut of *M. melolontha* (15). We developed a transformation technique for Da-11 that allowed the chromosomal insertion of a select-

* Corresponding author. Mailing address: School of Integrative Biology, The University of Queensland, St. Lucia, Queensland 4072, Australia. Phone: 61 7 3365 2471. Fax: 61 7 3346 9213. E-mail: scott.oneill@uq.edu.au.

▽ Published ahead of print on 2 May 2008.

able marker and quantified its establishment in the larval hindgut environment of *D. albohirtum* in a laboratory-based feeding trial.

MATERIALS AND METHODS

Bacterial growth conditions. *Escherichia coli* strain DH5α was grown on Luria broth agar plates or in Luria broth at 37°C. Da-11 was grown in 43 g liter^{-1} Wilkins-Chalgren anaerobe broth (WCAB; Oxoid, Adelaide, Australia) or on plates by adding 15 g liter^{-1} agar (Sigma-Aldrich, St. Louis, MO) to WCAB at 37°C. All liquid cultures were grown in an orbital shaker at 200 rpm. The antibiotic kanamycin was used at 50 μg/ml unless stated otherwise.

Sample collection. Third-instar larvae of the melolonthine scarab beetle *Dermolepida albohirtum* were collected from commercial sugarcane fields in three consecutive years in April. Larvae were collected from four regions approximately 200 to 300 km apart in northern Queensland, Australia, in 2005 and from a single region in 2006 and 2007. Once collected, larvae were kept separately in soil-free sterile containers before being processed.

Isolation of gut bacteria. Larvae from 2005 were kept for 7 or 45 days without food or water before dissection to reduce the occurrence of transient bacteria. Larvae were surface sterilized by immersion in sodium hypochlorite (50 g liter^{-1}) and rinsed with 70% ethanol, and the following procedures were carried out using standard sterile techniques. Larvae were dissected and the hindgut chamber isolated as described previously (32). The hindgut chamber was cut in half and rinsed three times in sterilized water to remove the lumen contents. The hindgut walls of two larvae collected from each of the four geographic regions were combined in a 1.5-ml reaction tube and ground using a pestle. Serial dilutions were made from the pooled hindgut wall tissue by use of 13 g liter^{-1} nutrient broth (Oxoid), and 50-μl volumes were spread evenly on agar plates containing the following media and incubated at 25°C under aerobic, microaerophilic (5 to 15% O_2, 5 to 12% CO_2), and anaerobic (0.1% O_2, 4 to 10% CO_2) conditions, created using a BBL GasPak system (BD, Sydney, Australia). Media types used were as follows (per liter): 28 g nutrient agar (Oxoid), 52 g reinforced clostridial agar (Oxoid), and 43 g WCAB (Oxoid) including 15 g agar (Sigma). All media types were used with different combinations of atmospheric conditions and additives (5% [vol/vol] defibrinated blood and 1 g liter^{-1} starch). Colonies that grew on agar plates from the highest serial dilutions after 2 weeks of incubation were picked and restreaked to obtain pure isolates. These isolates were assessed for the ability to grow in nutrient broth and WCAB and stored in 20% glycerol at −80°C.

Molecular identification and DNA-based DGGE analysis of isolates. Denaturing gradient gel electrophoresis (DGGE) analysis was used to rapidly identify different bacterial isolates sharing similar morphological characteristics, with potentially matching dominant bands, from hindgut wall samples of larvae collected in 2005. Cultured isolates were grown in liquid broth from −80°C stocks, and DNAs were extracted using a phenol-chloroform method (20). DNAs were extracted from complete individual larval hindgut wall samples by use of a PowerSoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA) following the manufacturer's protocol. Fragments of 16S rRNA genes were amplified from DNAs extracted from isolates and hindgut wall samples, using the DGGE universal primers F-968-GC and R-1401 (30) (Table 1). Isolates identified as unique bands in DGGE gels were sequenced using universal primers 61F and 1227R (31) (Table 1). For phylogenetic analysis, nearly full-length 16S rRNA gene sequences were obtained for Da-11, using universal primers 10F and 1500R (Table 1). Previously reported standard PCR conditions (32) were used for all PCR amplifications.

Mixed-species feeding trial. A feeding trial was designed to identify bacteria that could become established in the hindguts of larvae. Approximately 40 larvae collected in 2006 were kept for 3 to 7 days without food or water before commencement of the feeding trial. Eight species of bacteria that were first isolated from the hindguts of larvae in 2005 were used in combination for the feeding trial. The eight species of bacteria were independently grown to stationary phase in liquid broth, adjusted to a density of approximately 10^7 cells/ml, and then combined. Carrot pieces (2 cm^3) were immersed into the combined inoculum for 1 hour and then fed to individual larvae once. Control larvae were fed carrots without inoculum. Only larvae that ate the entire carrot piece within 2 days and remained healthy for the duration of the experiment were used for further analysis. The individual containers of larvae were cleaned every 2 days, and larvae were starved of food and water postinoculation for 19 days before being processed.

RNA-based DGGE analysis of inoculants. The hindgut chamber was isolated from 10 larvae collected in 2006 following the protocol mentioned above, except that the hindgut lumen was also collected. Total RNA extractions were carried out separately on individual larval hindgut lumen and wall samples, using a PowerSoil total RNA isolation kit (Mo Bio Laboratories) following the manufacturer's protocol. Approximately 0.4 g of gut sample was used in each RNA extraction mix, and several samples were discarded due to incomplete removal of environmental contaminants/enzyme inhibitors. Contaminating DNA was excluded from RNA extracts by using DNase (Invitrogen) digestion following the manufacturer's protocol and was checked by PCR using primers F-968 and R-1401 (Table 1). A second round of DNase digestion was needed for two samples. First-strand cDNA synthesis was carried out using primers F-968 and R-1401 and SuperScript III reverse transcriptase (Invitrogen), following the manufacturer's protocol. PCR amplification was carried out on cDNA under the conditions mention above, using F-968-GC and R-1402, followed by DGGE analysis.

TABLE 1. Sequences of primers used in this study

Primer	Sequence (5′ to 3′)	Reference
F-968-GC	CGCCCGGGGCGCGCCCCGGGCGG GGCGGGGGCACGGGGGGAACG CGAAGAACCTTAC	30
F-968	GAACGCGAAGAACCTTAC	30
R-1401	CGGTGTGTACAAGACCC	30
61F	GCTTAACACATGCAAG	31
1227R	CCATTGTAGCACGTGT	31
10F	AGTTTGATCCTGGCTCAG	This work
1500R	CTACCTTGTTACGACTTAGTCC	This work
T7	TAATACGACTCACTATAGGG	Promega
SqFP	GCCAACGACTACGCACTAGCCAAC	Epicentre
SqRP	GAGCCAATATGCGAGAACACCCG AGAA	Epicentre
3892F[a]	GAATTCTGTCAGCTACTGGGCTAT CTGG	This work
4944R[b]	GGATCCTCAGAAGAACTCGTCAAG AAGG	This work

[a] The underlined section of primer sequence corresponds to an EcoRI restriction site.
[b] The underlined section of primer sequence corresponds to a BamHI restriction site.

DGGE conditions. Heterogeneous 16S rRNA amplicons were separated using a previously reported DGGE protocol (32). The DNA-based DGGE gels used to screen isolates were visualized after being stained with ethidium bromide. The RNA-based DGGE gels used for the feeding trial were silver stained (37). Bands of interest were cut from the RNA-based DGGE gels, and DNA was eluted overnight at 4°C in 50 μl of water. Eluted DNAs were then reamplified and rerun in DGGE gels along with the original samples to check the accuracy of gel extraction procedures before sequencing.

Cloning, sequencing, and phylogenetic analysis. Cloning, sequencing, and phylogenetic analysis methods were carried out as described previously (32). Briefly, PCR products were cloned and sequenced and then sequence similarity searches were performed, using Seqmatch in the Ribosomal Database Project II (RDP II) (http://www.rdp.cme.msu.edu/) and BLASTN. For phylogenetic analysis, the 16S rRNA sequence (1,349-bp fragment) obtained from Da-11 was aligned with those of its nearest neighbors found in the RDP II. Trees constructed using maximum likelihood and Bayesian inference included the nucleotide substitution model TrN + I + G, where TrN is equal transversion and unequal transition rates and I is proportion of invariable sites.

Transposon construction. A section of plasmid pBBR1MCS-2 (pBBR) (26) from positions 3892 to 4944 that included the kanamycin resistance gene (*kanR*) was amplified with primers that also encoded restriction sites for EcoRI, at residue 3892F, and BamHI, at residue 4944R (Table 1). The PCR product was ligated into pGEM-T Easy (Promega, Madison, WI), electroporated into DH5α, and grown under kanamycin selection.

The multicloning site situated inside the EZ::Tn*5* transposable element on plasmid pMOD-2<MCS> (pMOD) (Epicentre, Madison, WI) was digested with EcoRI ((New England BioLabs [NEB], Beverly, MA) and BamHI (NEB) and ligated to the pBBR fragment containing *kanR* to form the transposable element EZ::Tn*5*-*kanR* in pMOD-2<MCS>, which was then tested for kanamycin resistance in DH5α. Plasmid pMOD-2<MCS> was then digested with PshAI (NEB), and the 1,227-bp fragment was gel purified using a MinElute gel extraction kit

(Qiagen, Hilden, Germany), following the manufacturer's protocol, to prepare it for electroporation.

Transposomes (Epicentre, Madison, WI) were generated according to the manufacturer's protocol. Briefly, 2 μl of EZ::Tn*5*-*kanR* (100 ng/μl), 4 μl of EZ::Tn*5* transposase (Epicentre), and 2 μl of 100% glycerol were mixed by vortexing and incubated at room temperature for 30 min. The transposome mix (1 μl) was then electroporated into Da-11 as mentioned below.

Transformation. A single Da-11 colony was picked and grown overnight in 50 ml of WCAB to an optical density at 600 nm (OD_{600}) of 0.7 cm^{-1}. Twenty milliliters of this culture was inoculated into 1 liter of fresh WCAB and grown to an OD of 0.9 cm^{-1}. Cells were pelleted at 1,560 × *g* for 12 min in a precooled (4°C) centrifuge and resuspended with an equal volume of chilled water. This procedure was carried out twice, then once with 500 ml of chilled 10% glycerol (filter sterilized). The cells were then pelleted at 2,515 × *g*, resuspended in 10% glycerol to a density of 10^9 cells/ml, and kept on ice until electroporation procedures commenced.

Electroporation was carried out using a BTX Electro 600 cell manipulator (Genetronics, San Diego, CA), 0.2-mm cuvettes, 5×10^8 cells (50 μl), a 50-μF capacitance, a 2.5-kV electrical potential difference, and resistance setting 9 (480 Ω), delivering current over 18 ms. Cells were mixed with DNA and incubated on ice for 30 min, and immediately after electroporation, they were mixed with 400 ml of SOC medium (36) and incubated for 2 h in an orbital shaker. Cells were then plated using kanamycin selection and checked for colonies after 3 days.

Plasmid and transposon analysis. Colonies of Da-11 that grew on kanamycin plates were screened for pBBR by PCR, followed by restriction fragment polymorphism analysis and then rescue of pBBR from Da-11 back into DH5α. Three days after electroporation, Da-11 colonies were selected from kanamycin plates, restreaked under the same conditions, and then picked for overnight growth in WCAB containing kanamycin. DNA was extracted from 4 ml of each colony by use of a phenol-chloroform method (20), followed by PCR detection of pBBR by using primers 3892F and 4944R, and plasmid was extracted from 5 ml of each colony by using a QIAprep Spin miniprep kit (Qiagen) following the manufacturer's protocol. Control pBBR and plasmids extracted from transformed colonies were digested using SfoI (NEB) or RsaI (NEB) and run in a 1% agarose gel for comparison. DH5α was electroporated with plasmid isolated from a Da-11 colony that was previously transformed with pBBR and grown on plates containing kanamycin.

Colonies of Da-11 that grew on kanamycin plates following transposome electroporation were screened for insertions of EZ::Tn*5*-*kanR* by PCR using primers 3892F and 4944R, Southern blotting, and finally, sequencing of insertion site flanking regions. Colonies were processed and DNA extracted using the methods described above for plasmid analysis.

To sequence the transposon-flanking regions, DNA was extracted from a PCR-positive colony, digested with Eco0109I (NEB), and ligated into plasmid pBluescript II SK (+) (Stratagene, La Jolla, CA). Two PCRs were carried out separately on pBluescript II SK (+) following the ligation reaction, using primers T7 and SqFP or T7 and SqRP (Table 1) to amplify the flanking regions of EZ::Tn*5*-*kanR*. The two PCR products were cloned and sequenced in one direction, using either SqFP or SqRP and BLASTN searches carried out on the sequenced fragments.

The random chromosomal insertion of EZ::Tn*5*-*kanR* into Da-11 colonies was inferred by Southern blotting analysis carried out according to the methods of Sambrook et al. (36). DNAs were digested from PCR-positive and wild-type colonies by use of EcoRV (NEB), transferred to a nylon membrane (Hybond-XL; Amersham Biosciences, Sweden) following the manufacturer's protocol for neutral transfer, and hybridized with a ^{32}P-labeled probe made from the EZ::Tn*5*-*kanR* transposon, using reagents and the standard protocol from a random primed DNA labeling kit (Roche, Mannheim, Germany). After overnight probe hybridization, the membrane was washed under four increasingly stringent conditions, ranging from 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate to 0.1× SSC and 0.1% sodium dodecyl sulfate (36). Hybridization patterns were captured and processed using a Storm 830 phosphorimager and ImageQuant TL software (Amersham Biosciences).

Transgenic feeding trial. Larvae collected from the field in 2007 were used in a second feeding trial that repeated the methods used for larvae collected in 2006. Five Da-11 colonies with unique EZ::Tn*5*-*kanR* genomic insertions were grown separately overnight without kanamycin selection. The five unique transgenic colonies were combined and serially diluted using WCAB to make three inocula, at 1×10^7, 1×10^5, and 1×10^3 cells/ml, in a final volume of 100 ml each. Fifty carrot pieces (~2 cm^3) were immersed into one of the three inocula, and then single carrot pieces were fed to individual larvae once. We gained an estimate of the average number of cells on each carrot piece by measuring the amount of fluid displaced when 50 carrot pieces were removed from each 100-ml inoculum. Only larvae that ate the entire carrot piece within 2 days were used for further analysis. Larvae were processed at days 9 and 21 postinoculation. The entire hindgut chamber was removed from surface-sterilized larvae collected in 2007, placed in a 1.5-ml reaction tube with 500 ml of WCAB with 100 μg/ml kanamycin, and ground using a pestle. Serial dilutions (1:100, 1:1,000, 1:10,000, and 1:100,000) were made from individual hindgut samples, spread evenly on agar plates containing WCAB and 200 μg/ml of kanamycin, and incubated at 37°C for 3 days.

The hindgut contents of larvae fed carrot with and without a transgenic inoculum were plated. The latter was used to identify microbial species from the hindgut environment that were naturally resistant to kanamycin, whereas transgenic Da-11 colonies were identified by time of colony emergence and colony morphology and color. A subset of colonies identified as Da-11 were subjected to further verification by microscopic morphology, restreaking, and PCR detection of the EZ::Tn*5*-*kanR* insert, using primers 3892F and 4944R. Of the 46 colonies checked, one had unusual morphology and failed the PCR screen; as a result, a 2% error rate was factored into density estimates. The number of Da-11 cells per hindgut was then estimated by calculating the average volume of a third-instar hindgut by grinding the hindgut chamber of three larvae in 1.5-ml reaction tubes and noting the volume. The total number of microbes present in the hindguts of three larvae was estimated using a cell counter and microscope.

Da-11 type strain. Isolate Da-11 is publicly available from the Australian Collection of Microorganisms under accession number ACM 5246.

Nucleotide sequence accession numbers. Nucleotide sequences were deposited in the GenBank database under accession numbers EU073949 to EU073961, corresponding to isolates Da-10 to Da-23, respectively.

TABLE 2. Isolated bacteria from the hindgut wall of *Dermolepida albohirtum* larvae and the nearest type strains found using RDP II

Isolate[a]	Phylogenetic group	% Similarity[b]	Nearest neighbor
Da-10*	*Alphaproteobacteria*	99	*Brevundimonas vesicularis*
Da-11*	*Betaproteobacteria*	93	*Aquabacterium citratiphilum*
Da-12		99	*Alcaligenes faecalis*
Da-13*	*Gammaproteobacteria*	99	*Enterobacter ludwigii*
Da-14		99	*Citrobacter werkmanii*
Da-15		99	*Serratia marcescens*
Da-16		99	*Stenotrophomonas maltophilia*
Da-17*	*Firmicutes*	99	*Lactococcus garvieae*
Da-18*		96	*Lactococcus lactis*
Da-19		99	*Enterococcus gallinarum*
Da-20		99	*Enterococcus avium*
Da-21*	*Actinobacteria*	97	*Microbacterium ellin*
Da-22*		99	*Microbacterium ellin*
Da-23*		98	*Microbacterium phyllosphaerae*

[a] *, isolates used in feeding trial (Fig. 1).
[b] Over a 1,166-bp fragment of the 16S rRNA gene.

RESULTS

DNA-based DGGE analysis of isolates and gut profiles. DGGE analysis was used to compare 50 colonies cultured from the hindgut wall of *D. albohirtum* larvae to hindgut wall bacterial community profiles (data not shown). Twenty-five colonies were selected from larvae that were starved of food and water for either 7 or 45 days. Although a range of culturing conditions was used, the bacteria isolated in culture were not the most common gut-associated bacteria identified in DGGE hindgut profiles (32). Fourteen isolates possessing unique DGGE bands were amenable to culturing techniques, having a doubling time of less than 3 h in either nutrient broth or

WCAB. These 14 unique colonies, presented in Table 2, were isolated from larvae starved for 45 days.

Identification of isolates. Based on 16S rRNA gene classification, the 14 bacterial species isolated from the hindgut wall of *D. albohirtum* larvae were in the phyla *Proteobacteria*, *Firmicutes*, and *Actinobacteria*. Similarly, unculturable taxa from these three phyla were predominant in DGGE larval hindgut wall profiles for *D. albohirtum* (32) and from the molecular characterization of the larval guts of *Melolontha melolontha* (15) and *Pachnoda ephippiata* (16). RDP II was used to search for the closest cultured type strains (Table 2). Of the 14 isolates selected for sequence comparisons, 3 were potentially new species, sharing <97.5% similarity of the 16S rRNA gene to the closest type strain relative. Type strains were typically from rhizosphere environments and the intestines of invertebrates. *Microbacterium phyllosphaerae*, isolated from the phyllosphere of grass (4), closely matched Da-23 (Table 2). *Alcaligenes faecalis* and *Stenotrophomonas maltophilia* are associated with the soil microarthropod *Folsomia candida* (40) and matched Da-12 and Da-16, respectively. *A. faecalis* and *S. maltophilia* have been shown to become established in the gut of *F. candida* in laboratory-based feeding trials (40). Da-15 matched *Serratia marcescens*, a commonly found intestinal species in many invertebrates. However, *S. marcescens* failed to become established among the resident hindgut communities of three termite species in laboratory-based feeding trials (43). Da-13 matched *Enterobacter ludwigii*. This genus includes *Enterobacter cloacae*, which has been included in research for potential paratransgenic approaches to control termites (22) and corn borer and cotton bollworm larvae (39); *Enterobacter agglomerans*, studied to control grasshoppers (9); and *Enterobacter gergoviae*, targeting pink bollworm (27).

The inclusion of uncultured taxa from the RDP II database revealed that Da-11 matched closely with MKEW-111 (accession no. AJ852369), found in the gut of *M. melolontha* (15), and PeM24 (accession no. AJ576389) and PeM09 (accession no. AJ576380), from *P. ephippiata* larvae (16). Da-21 matched closely with PeM73 (accession no. AJ576426), also from *P. ephippiata* larvae. Based on this phylogenetic information, a subset of bacteria from the taxa *Firmicutes*, *Proteobacteria*, and *Actinobacteria*, shown in Table 2, were selected for further assessment as potential delivery vehicles of antifeeding molecules for the control of *D. albohirtum* larvae.

RNA-based DGGE analysis of inoculants. A feeding trial was designed to identify cultured isolates capable of becoming established within the hindguts of *D. albohirtum* larvae. DGGE analysis was used to detect the presence of eight cultured isolates fed to larvae among the resident gut community. To infer metabolic activity, rRNA from the 16S rRNA gene was used instead of DNA. Among the two control and four treatment larvae screened using this approach, Da-11 (Table 2) was the only species detected at a significant density, in the hindgut of a single larva, after a 19-day period (Fig. 1). Consequently, a 1,349-bp fragment of the 16S rRNA gene was sequenced from Da-11 and used to construct maximum likelihood (not shown) and Bayesian inference (Fig. 2) phylogenetic trees. Da-11 is from the order *Burkholderiales* and was found to share 93% similarity with type strains from a range of genera, including *Aquabacterium*, *Rubrivivax*, and *Schlegelella*. Both phylogenetic trees placed Da-11 as a deep branch of the

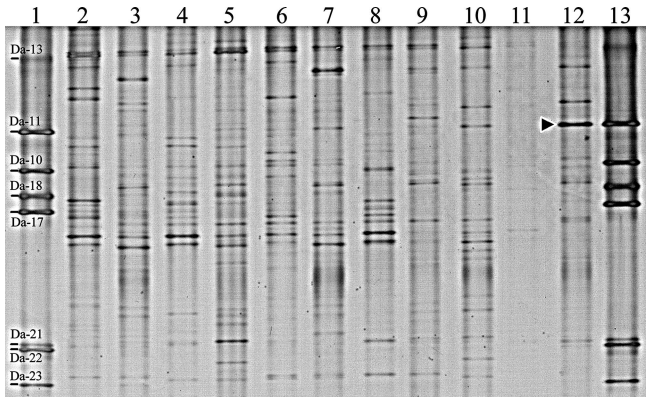

FIG. 1. 16S rRNA DGGE profiles of bacterial communities associated with the hindgut wall (lanes 3, 5, 7, 9, 10, and 12) and lumen (lanes 2, 4, 6, 8, and 11) of *Dermolepida albohirtum* larvae. DGGE analysis was used to identify cultured isolates (lanes 1 and 13) that were inoculated onto carrot and fed to larvae (lanes 6 to 12); controls (lanes 2 to 5) were fed carrot pieces without inoculum. All larvae were processed at 19 days postinoculation. The numbers in lane 1 correspond to isolates presented in Table 2. The arrow in lane 12 shows the band that was sequenced and matched to Da-11.

Schlegelella genus. The closest noncultured clones were found inhabiting the hindgut wall of *M. melolontha* larvae (MKEW-111) (15) and the midgut of *P. ephippiata* larva (PeM24 and PeM09) (16). MKEW-111 shared 99% similarity with Da-11 across the 1,349-bp fragment of the 16S rRNA gene (Fig. 2).

Transformation efficiency of Da-11. We were successful in transforming Da-11 with the broad-host-range plasmid pBBR. Plasmid pBBR was chosen for these initial attempts because it was originally isolated from *Bordetella bronchiseptica*, which is also from the order *Burkholderiales* (1). Colonies of Da-11 that were electroporated with pBBR were visible after 3 days of incubation on WCAB agar plates containing kanamycin (50 μg/ml). Colony formation was not detected when untransformed controls were incubated on WCAB agar plates containing kanamycin (20 to 50 μg/ml), suggesting that Da-11 is very susceptible to this antibiotic.

Plasmid and transposon analysis. The acquisition of pBBR by Da-11 was detected by PCR amplification of *kanR* present on pBBR, as wild-type colonies did not produce an appropriately sized band. The restriction fragment length polymorphism patterns created using SfoI or RsaI on control pBBR and plasmid extracted from Da-11 colonies transformed with pBBR were identical. Finally, transformation of DH5α with plasmid extracted from a Da-11 colony resulted in kanamycin-resistant colonies.

Since plasmid pBBR produced Da-11 colonies that were resistant to kanamycin, a section of plasmid pBBR containing *kanR* was inserted inside the transposable element EZ::Tn*5*, located on plasmid pMOD-2<MCS>, to construct the transposable element EZ::Tn*5*-*kanR*. When 5×10^8 cells of Da-11 (50 μl) were electroporated with 25 ng of EZ::Tn*5*-*kanR* (1 μl of transposome) and plated on WCAB agar containing kanamycin (20 μg/ml), approximately 1,000 cells were visible after 3 days of growth. Repeated attempts using EZ::Tn*5*-*kanR* (25 ng) gave similar results, suggesting that the transformation

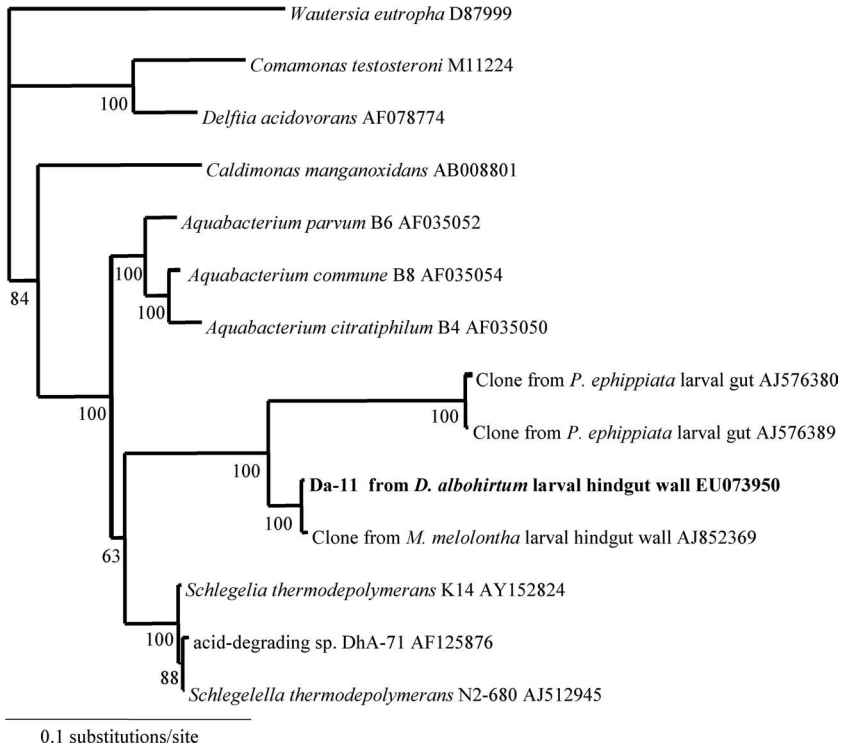

FIG. 2. Phylogenetic relationships of Da-11 (in bold), isolated from the hindgut wall of *Dermolepida albohirtum* larvae, and the closest noncultured and cultured relatives found using the Ribosomal Database Project II (accession numbers are indicated on the branches). Tree construction used Bayesian analysis of nearly full-length 16S rRNA gene sequences, with Bayesian posterior probabilities indicated at each node.

efficiency of Da-11 is approximately 4×10^4 CFU/μg of transposon DNA.

All 10 randomly picked Da-11 colonies that were transformed with EZ::Tn*5*-*kanR* displayed PCR products of equal length to the control transposon. Genomic DNA digested with a restriction enzyme that cuts outside the transposon was used for Southern blot analysis of four Da-11-transformed colonies and a control (Fig. 3). The four transformed colonies had bands of unequal length, while the wild-type colony did not produce a band when probed with radiolabeled EZ::Tn*5*-*kanR* (Fig. 3).

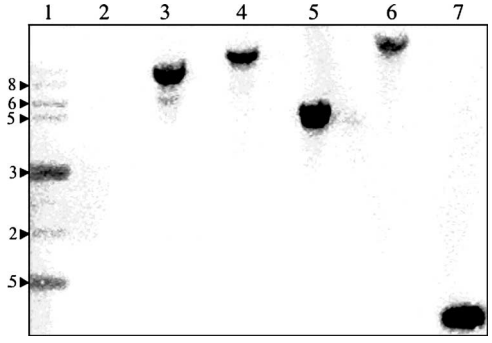

FIG. 3. Southern blot analysis of digested genomic DNAs from Da-11 colonies transformed with the transposable element EZ::Tn*5*-*kanR*. Lane 2, wild-type colony; lanes 3 to 6, individual colonies transformed with EZ::Tn*5*-*kanR*; and lane 7, EZ::Tn*5*-*kanR*. The membrane was probed with radiolabeled EZ::Tn*5*-*kanR*. Arrows to the left indicate molecular size standards (kilobases).

The flanking regions of EZ::Tn*5*-*kanR* and genomic DNA bordering the insertion site were sequenced for a single transformed Da-11 colony. An 832-bp genomic fragment connecting the leading flanking region of EZ::Tn*5*-*kanR* and a 676-bp genomic fragment connecting the trailing flanking region were sequenced. The junctions connecting genomic DNA and the leading and trailing ends of EZ::Tn*5*-*kanR* had the sequences 5′-AAAGTAAAACCTGTCTCTTATACACATCT-3′ and 5′-GATGTGTATAAGAGACAGGTAAAAAAC GG-3′, respectively. The underlined sections are the EZ::Tn*5*-*kanR* insert ends, and the nonunderlined sections are genomic DNA from Da-11. BLASTN searches of the 832-bp and 676-bp fragments found no full-length matches to any genomes in the NCBI database.

Quantification in larval hindgut. Da-11 cells transformed with EZ::Tn*5*-*kanR* were rescued from the hindguts of larvae at 9 and 21 days postconsumption. Three separate groups of 50 individual larvae were given either a high (4×10^6 cells), medium (4×10^4 cells), or low (4×10^2 cells) dose of Da-11 that incorporated an EZ::Tn*5*-*kanR* genomic insert. The three doses of cells were inoculated onto pieces of carrot, which were then fed to larvae. Over 20 individuals from each group had eaten the entire inoculated carrot piece within 2 days, and subsequently only these larvae were used for further analysis.

Plate counts of larval hindgut contents showed that at 9 days postconsumption, 40% (6 of 15 larvae), 60% (9 of 15 larvae), and 61% (8 of 13 larvae) of individuals from the low-, medium-, and high-dose treatment groups, respectively, had detectable levels of Da-11. At 21 days postconsumption, the infection

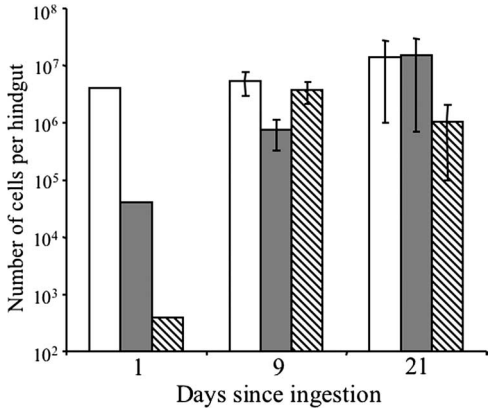

FIG. 4. Abundance of transgenic Da-11 cells obtained in the hindguts of third-instar larvae of *Dermolepida albohirtum* at 9 and 21 days postconsumption. The standard error bars around the means are given for three inocula of cells inoculated onto carrot pieces and fed to larvae over a 2-day period. Day 1 columns show the three doses ingested by larvae (nonshaded bars, 4×10^6 cells; shaded bars, 4×10^4 cells; and striped bars, 4×10^2 cells). A logarithmic scale for cells per hindgut was used.

frequency increased to 50% (4 of 8 larvae) for low-dose treatment and 75% (6 of 8 larvae) for high-dose treatment, and the frequency for the medium-dose treatment remained at 57% (4 of 7 larvae) of individuals having detectable levels of Da-11. Only larvae with detectable levels of Da-11 were included in the following average density estimates (Fig. 4).

At 9 days postconsumption, the average density of transgenic Da-11 cells reached 3×10^6 cells/hindgut from an ingested amount of ~400 cells (Fig. 4). When the starting amount of Da-11 was increased 100- and 10,000-fold, similar average densities were found, at 7×10^5 and 5×10^6 cells/hindgut, respectively. After 21 days, Da-11 had increased to 1×10^7 cells/hindgut for medium and high doses and reduced to 1×10^6 cells/hindgut for low doses (Fig. 4).

The total number of cells present in the hindgut, as determined by microscopic cell counts for three larvae, ranged from 2×10^{10} to 3×10^{10} cells/ml, and the volumes of hindgut chambers ranged from 0.9 to 1.2 ml. Hence, converting density estimates from cells/hindgut to cells/ml is approximately equivalent for third-instar larvae of *D. albohirtum*.

DISCUSSION

The hindgut of the *D. albohirtum* larva houses a diverse microbial community, including transiently acquired species and bacteria that are closely associated with the host (15, 16, 32). Bacteria that can be acquired by larvae during feeding and remain metabolically active in significant densities in the gut environment are potential candidates as transgenic delivery vehicles (32). Hence, our first goal in this study was to isolate and identify bacteria that can be passaged through the intestinal system of scarab larvae and be maintained in the complex yet stable hindgut environment.

The diverse microbial community that exists in the gut lumen of root-feeding scarab larvae is relatively transient compared to the hindgut wall community, which has taxa that are found consistently across geographic regions (15, 32). We predicted that species capable of adhering to the hindgut wall might persist in this environment for prolonged periods. Subsequently, our study focused on isolating bacteria associated with the hindgut walls of larvae. We were able to isolate Da-11 on general media and showed that it is capable of becoming established in the intestinal system and remaining metabolically active among the members of the hindgut wall microbial community for prolonged periods. Interestingly, in the initial feeding trial (Fig. 1), Da-11 was not detected in the lumen, consistent with research that detected this species only on the larval hindgut wall of the European organism *M. melolontha* (15).

A number of methods were combined to improve the likelihood of isolating species adapted to a specific niche in the gut environment, rather than transients acquired by larvae during feeding. Culturing methods included pooling larvae from multiple geographic regions before serial dilution, selecting media that promote the growth of bacteria identified in previous research (32), and starving larvae for prolonged periods. Unfortunately, we were unable to isolate the dominant species identified in DGGE gut profiles under a range of general culturing conditions.

Promicromonospora pachnodae, from the phylum *Actinobacteria*, is a member of the hindgut community of larvae of *Pachnoda marginata* (8). We discovered three *Actinobacteria* isolates from the *Microbacterium* genus. This genus has taxa identified from the midguts of scarab (16) and gypsy moth (5) larvae; *Microbacterium nematophilum*, known to cause intestinal inflammation in nematodes (19); isolates found degrading pesticides in soil (12); and endophytes isolated from agronomic crops targeted for crop protection (44). However, our three *Microbacterium* isolates could not be detected in the hindgut in our initial feeding trial (Fig. 1), indicating that they may be of limited utility in a future paratransgenic approach.

Similarly, members of the order *Burkholderiales* are increasingly being utilized in agricultural applications due to their diverse roles within rhizosphere communities (10). However, many of these isolates are closely related to the *Burkholderia cepacia* complex involved in human infection. This potential negative impact has prompted researchers to isolate plant-beneficial bacteria from the *Burkholderia* genus that are phylogenetically distant from this clade (6). Fortunately, Da-11 is distant from the *B. cepacia* clade, branching deeply from the closest classified genera, and potentially forms a new genus in the *Burkholderiales* order, along with taxa from other scarabs.

Despite the phylogenetic placement of the isolates in Table 2 as potential candidates for the paratransgenic control of scarab larvae, our DNA-based DGGE gut profiles and RNA-based DGGE feeding trial results suggested otherwise. Only one of eight species was significantly active in the larval hindgut of *D. albohirtum*, confirming research suggesting that the gut environment of many soil invertebrates is a selective habitat for microorganisms (23, 40, 43). To our knowledge, Da-11 is the only species isolated from our study that has not been detected in any other environment besides the guts of scarab larvae to date. This provides additional evidence that Da-11 is adapted to this particular environment.

Ultimately, our aim is to control the feeding damage caused by scarabs in agricultural crops by expressing transgenic antifeeding compounds from bacteria in the guts of larvae. Using

bacteria that are adapted to the gut environment of a specific insect host will potentially increase exposure to effector molecules. Hence, our second goal in this study was to develop a protocol for the stable insertion of transgenes into the chromosomes of bacteria that are adapted to the hindgut environment of scarab beetle larvae. We showed that transgenic Da-11 could become reestablished in this environment among the full spectrum of indigenous microbes and could maintain its antibiotic marker in the absence of selection for the 21 days of the experiment.

Improved delivery of antifeeding compounds may be accomplished by transforming microbial species that can remain metabolically active at high densities for prolonged periods in the gut environment. In scarab larvae, infection frequencies may be restricted by harsh midgut conditions (28, 29), competitive interactions with resident microorganisms in the hindgut (15, 16, 32), an inability to grow fast enough to replace cells lost with the passage of food, or an inability to attach to the gut wall. The total density of bacteria within the hindgut chamber of scarab larvae has been estimated for four species (2, 7, 15, 28). These estimates range from 9×10^9 to 5×10^{10} cells/ml, and our estimates for *D. albohirtum* larvae fall within this range. Molecular surveys from two scarab species estimate that between 60 and 200 bacterial species are present in the larval hindgut at any one time (15, 16). A crude estimate, ignoring skewed species distributions, places the average density of a single bacterial species in the hindgut of a scarab larva at between 1×10^7 and 1×10^9 cells/ml. Similarly, *Promicromonospora pachnodae* isolated from the larval hindgut of *Pachnoda marginata* was estimated by plate counts at 2.5×10^8 to 7.4×10^8 cells/ml (8), and sulfate-reducing bacteria from the genus *Desulfovibrio* reached 2×10^8 cells/ml (13).

We found that Da-11 was able to become established in the larval hindgut of *D. albohirtum* and to maintain a density of 1×10^6 cells/ml from a starting oral dose of only 4×10^2 cells. This suggests that Da-11 is adapted to the hindgut environment, although perhaps not as a dominant hindgut member. This is congruent with unsuccessful attempts to identify Da-11 among the dominant members in hindgut DGGE profiles (32). Overall, our results suggest that once inside the hindgut environment, Da-11 replicates to densities high enough to place it as a candidate for further development for the delivery of effector molecules targeting the feeding behavior of scarab larvae.

ACKNOWLEDGMENTS

We thank Bill Harris, Mohamed Sallam, and Peter Samson for assistance in locating and collecting scarab larvae; members of the O'Neill laboratory, particularly Inaki Iturbe-Ormaetxe and Jeremy Brownlie, for technical support; Beth McGraw for phylogenetic advice; and Hongyu Zhang and Trevor Jackson for advice on DGGE analysis.

This work was supported by an Australian Research Council grant.

REFERENCES

1. **Antoine, R., and C. Locht.** 1992. Isolation and molecular characterization of a novel broad-host-range plasmid from *Bordetella bronchiseptica* with sequence similarities to plasmids from gram-positive organisms. Mol. Microbiol. **6:**1785–1799.
2. **Bauchop, T., and R. T. J. Clarke.** 1975. Gut microbiology and carbohydrate digestion in the larva of *Costelytra zealandica* (Coleoptera: Scarabaeidae). N. Z. J. Zool. **2:**237–243.
3. **Beard, B. C., S. L. O'Neill, R. B. Tesh, F. F. Richards, and S. Aksoy.** 1993. Modification of arthropod vector competence via symbiotic bacteria. Parasitol. Today **9:**179–183.
4. **Behrendt, U., A. Ulrich, and P. Schumann.** 2001. Description of *Microbacterium foliorum* sp. nov. and *Microbacterium phyllosphaerae* sp. nov., isolated from the phyllosphere of grasses and the surface litter after mulching the sward, and reclassification of *Aureobacterium resistens* (Funke et al. 1998) as *Microbacterium resistens* comb. nov. Int. J. Syst. Evol. Microbiol. **51:**1267–1276.
5. **Broderick, N. A., K. F. Raffa, R. M. Goodman, and J. Handelsman.** 2004. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Appl. Environ. Microbiol. **70:** 293–300.
6. **Caballero-Mellado, J., J. Onofre-Lemus, P. Estrada-de los Santos, and L. Martinez-Aguilar.** 2007. The tomato rhizosphere, an environment rich in nitrogen-fixing *Burkholderia* species with capabilities of interest for agriculture and bioremediation. Appl. Environ. Microbiol. **73:**5308–5319.
7. **Cazemier, A. E., J. H. P. Hackstein, H. J. M. Op den Camp, J. Rosenberg, and C. van der Drift.** 1997. Bacteria in the intestinal tract of different species of arthropods. Microb. Ecol. **33:**189–197.
8. **Cazemier, A. E., J. C. Verdoes, F. A. G. Reubsaet, J. H. P. Hackstein, C. van der Drift, and H. J. M. Op den Camp.** 2003. *Promicromonospora pachnodae* sp. nov., a member of the (hemi)cellulolytic hindgut flora of larvae of the scarab beetle *Pachnoda marginata.* Antonie van Leeuwenhoek **83:**135–148.
9. **Chapco, W., and R. A. Kellin.** 1994. Persistence of ingested bacteria in the grasshopper gut. J. Invertebr. Pathol. **64:**149–150.
10. **Chiarini, L., A. Bevivino, C. Dalmastri, S. Tabacchioni, and P. Visca.** 2006. *Burkholderia cepacia* complex: health hazards and biotechnological potential. Trends Microbiol. **14:**277–286.
11. Reference deleted.
12. **Desaint, S., A. Hartman, N. R. Parekh, and J. Fournier.** 2000. Genetic diversity of carbofuran-degrading soil bacteria. FEMS Microbiol. Ecol. **34:** 173–180.
13. **Droge, S., U. Limper, F. Emtiazi, I. Schonig, N. Pavlus, O. Drzyzga, U. Fischer, and H. Konig.** 2004. In vitro and in vivo sulfate reduction in the gut contents of the termite *Mastotermes darwiniensis* and the rose-chafer *Pachnoda marginata*. Eur. J. Entomol. **103:**779–786.
14. **Durvasula, R. V., A. Gumbs, A. Panackal, O. Kruglov, S. Aksoy, R. B. Merrifield, F. F. Richards, and C. B. Beard.** 1997. Prevention of insect-borne disease: an approach using transgenic symbiotic bacteria. Proc. Natl. Acad. Sci. USA **94:**3274–3278.
15. **Egert, M., U. Stingl, L. D. Bruun, B. Pommerenke, A. Brune, and M. W. Freidrich.** 2005. Structure and topology of microbial communities in the major gut compartments of *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. **71:**4556–4566.
16. **Egert, M., B. Wagner, T. Lemke, A. Brune, and M. W. Friedrich.** 2003. Microbial community structure in midgut and hindgut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. **69:**6659–6668.
17. **Favia, G., I. Ricci, C. Damiani, N. Raddadi, E. Crotti, M. Marzorati, A. Rizzi, R. Urso, L. Brusetti, S. Borin, D. Mora, P. Scuppa, L. Pasqualini, E. Clementi, M. Genchi, S. Corona, I. Negri, G. Grandi, A. Alma, L. Kramer, F. Esposito, C. Bandi, L. Sacchi, and D. Daffonchio.** 2007. Bacteria of the genus *Asaia* stably associate with *Anopheles stephensi*, an Asian malarial mosquito vector. Proc. Natl. Acad. Sci. USA **104:**9047–9051.
18. **Hillman, J. D.** 2002. Genetically modified *Streptococcus mutans* for the prevention of dental caries. Antonie van Leeuwenhoek **82:**361–366.
19. **Hodgkin, J., P. E. Kuwabara, and B. Corneliussen.** 2000. A novel bacterial pathogen, *Microbacterium nematophilum*, induces morphological change in the nematode *C. elegans*. Curr. Biol. **10:**1615–1618.
20. **Holmes, D. S., and J. Bonner.** 1973. Preparation, molecular weight, base composition, and secondary structure of giant nuclear ribonucleic acid. Biochemistry **12:**2330–2338.
21. Reference deleted.
22. **Husseneder, C., and J. K. Grace.** 2005. Genetically engineered termite gut bacteria (*Enterobacter cloacae*) deliver and spread foreign genes in termite colonies. Appl. Genet. Mol. Biotechnol. **68:**360–367.
23. **Husseneder, C., J. K. Grace, and D. E. Oishi.** 2005. Use of genetically engineered *Escherichia coli* to monitor ingestion, loss, and transfer of bacteria in termites. Curr. Microbiol. **50:**119–123.
24. **Irvin, N., M. S. Hoddle, D. A. O'Brochta, B. Carey, and P. W. Atkinson.** 2004. Assessing fitness costs for transgenic *Aedes aegypti* expressing the GFP marker and transposase genes. Proc. Natl. Acad. Sci. USA **101:**891–896.
25. **Jackson, T. A., J. F. Pearson, M. O'Callaghan, H. K. Mahanty, and M. J. Willocks.** 1992. Pathogen to product—development of *Serratia entomophila* (Enterobacteriaceae) as a commercial biological control agent for the New Zealand grass grub (*Costelytra zealandica*), p. 191–198. *In* T. A. Jackson and T. R. Glare (ed.), Use of pathogens in scarab pest management. Intercept, Andover, Hampshire, United Kingdom.
26. **Kovach, M. E., P. E. Elzer, D. S. Hill, G. T. Roberston, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
27. **Kuzina, L. V., E. D. Miller, B. Ge, and T. A. Miller.** 2002. Transformation of

Enterobacter gergoviae isolated from pink bollworm (Lepidoptera: Gelechiidae) gut with *Bacillus thuringiensis* toxin. Curr. Microbiol. **44:**1–4.
28. **Lemke, T., U. Stingl, M. Egert, M. W. Friedrich, and A. Brune.** 2003. Physiochemical conditions and microbial activities in the highly alkaline gut of the humus-feeding larva of *Pachnoda ephippiata* (Coleoptera: Scarabaeidae). Appl. Environ. Microbiol. **69:**6650–6658.
29. **McGhie, T. K., J. T. Christeller, R. Ford, and P. G. Allsopp.** 1995. Characterization of midgut proteinase activities of white grubs: *Lepidiota noxia*, *Lepidiota negatoria* and *Antitrogus consanguineus* (Scarabaeidae, Melolonthini). Arch. Insect Biochem. Physiol. **28:**351–363.
30. **Nubel, U., B. Engelen, A. Felske, J. Snaidr, A. Wieshuber, R. I. Amann, W. Ludwig, and H. Backhaus.** 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. **178:**5636–5643.
31. **O'Neill, S. L., R. Giordano, A. M. E. Colbert, T. L. Karr, and H. M. Roberston.** 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with the cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA **89:**2699–2702.
32. **Pittman, G. W., S. M. Brumbley, P. G. Allsopp, and S. L. O'Neill.** 2008. *Endomicrobia* and other bacteria associated with the hindgut of *Dermolepida albohirtum* larvae. Appl. Environ. Microbiol. **74:**762–767.
33. Reference deleted.
34. **Robertson, L. N., P. G. Allsopp, K. J. Chandler, and R. T. Mullins.** 1995. Integrated management of canegrubs in Australia: current situation and future directions. Aust. J. Agric. Res. **46:**1–16.
35. **Robinson, A. S., G. Franz, and P. W. Atkinson.** 2004. Insect transgenesis and its potential role in agricultural and human health. Insect Biochem. Mol. Biol. **34:**113–120.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37. **Sanguinetti, C. J., E. D. Neto, and A. J. G. Simpson.** 1994. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. BioTechniques **17:**914–921.
38. Reference deleted.
39. **Tang, C., F. Sun, X. Zhang, T. Zhao, and J. Qi.** 2004. Transgenic ice nucleation-active *Enterobacter cloacae* reduces cold hardiness of corn borer and cotton bollworm larvae. FEMS Microbiol. Ecol. **51:**79–86.
40. **Thimm, T., A. Hoffman, H. Borkott, J. C. Munch, and C. C. Tebbe.** 1998. The gut of the soil microarthropod *Folsomia candida* (Collembola) is a frequently changeable but selective habitat and a vector for microorganisms. Appl. Environ. Microbiol. **64:**2660–2669.
41. Reference deleted.
42. **van Elsas, J. D., A. C. Wolters, C. D. Clegg, H. M. Lappin-Scott, and J. M. Anderson.** 1994. Fitness of genetically modified *Pseudomonas fluorescens* in competition for soil and root colonization. FEMS Microbiol. Ecol. **13:**259–272.
43. **Vievers, P. C., R. W. O'Brien, and M. Slaytor.** 1982. Role of bacteria in maintaining the redox potential in the hindgut of termites and preventing entry of foreign bacteria. J. Insect Physiol. **28:**947–951.
44. **Zinniel, D. K., P. Lambrecht, N. B. Harris, Z. Feng, D. Kuczmarski, P. Higley, C. A. Ishimaru, A. Arunakumari, R. G. Barletta, and A. K. Vidaver.** 2002. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. Appl. Environ. Microbiol. **68:**2198–2208.