Morganella morganii (Enterobacteriales: Enterobacteriaceae) Is a Lethal Pathogen of Mexican Fruit Fly (Diptera: Tephritidae) Larvae

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Source: Florida Entomologist, 100(4) : 743-751

Published By: Florida Entomological Society

URL: https://doi.org/10.1653/024.100.0422

Morganella morganii (Enterobacteriales: Enterobacteriaceae) is a lethal pathogen of Mexican fruit fly (Diptera: Tephritidae) larvae

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Abstract

Tephritid pests, such as the Mexican fruit fly, *Anastrepha ludens* (Loew), represent a major threat to fruit production worldwide. In order to control these pests, sterile insect technique is used to suppress and eradicate wild populations. For this control method to be successful, hundreds of millions of flies must be produced weekly in mass rearing facilities. The large quantity of artificial diet and close proximity of flies at various life stages allows bacteria from family Enterobacteriaceae, Bacillaceae, Pseudomonadaceae, and others to multiply and spread more easily. In this study, bacteria with a possible pathogenic effect were isolated from Mexican fruit fly eggs and dead Mexican fruit fly larvae. Two strains of bacteria associated with dead and dying larvae were identified using the 16S rRNA sequence as a species of *Morganella*. Further sequencing of multiple genes and the entire genomes identified both strains as *Morganella morganii*. Pathogenicity tests were completed to assess this bacterium as a Mexican fruit fly pathogen. Several measures of pathogenicity including effects on larval and pupal weight, adult percent emergence, and flight ability were measured for the 2 strains of *Morganella* compared against a control. In all cases, the presence of the *Morganella* strains significantly reduced all quality control measurements compared to the control. Also, at 10^5 colony forming units per ml or higher levels of inoculum, the presence of *Morganella* resulted in 100% mortality of larvae. This study illustrates that *Morganella morganii* is an extremely lethal pathogen of mass reared Mexican fruit flies.

Key Words: mass rearing; sterile insect technique; *Anastrepha*; pathogenicity

Resumen

Las moscas tefritidas como la mosca Mexicana de la fruta (*Anastrepha ludens* Loew), representan un peligro para la producción de la fruta a nivel mundial. Para controlar estas plagas, la técnica del insecto estéril es usada para suprimir y erradicar poblaciones naturales. Para que este método de control tenga éxito, cientos de millones de moscas deben ser producidos en plantas de crianza masiva. La gran cantidad de dieta artificial y la cercanía de moscas en diversos estadios de desarrollo permiten que las bacterias perteneciendo a la familia Entobacteriacea, Baciliiacea, Pseudomonadacea, y otros se multipliquen y diseminen rápidamente. En este estudio, bacterias con posibilidad de ser patogénicas fueron aislados de huevos y larvas muertas de la mosca Mexicana de la fruta. Dos cepas de bacteria aisladas de larvas muertas o moribundas fueron identificados usando secuencias de ARN ribosoma 16S como una especie de *Morganella*. Adicionalmente, la realización de secuencias de múltiples genes y el genoma entero reveló que las dos cepas de bacteria fueron identificados como *Morganella morganii*. Pruebas de patogenicidad fueron completadas para determinar la patogenicidad de *Morganella* hacia la mosca Mexicana de la fruta. Diferentes variables de patogenicidad como el efecto sobre el peso de las larvas y pupas, porcentaje de emergencia de moscas adultas y habilidad de vuelo fueron evaluadas para las cepas de dos *Morganella* y comparados contra el control. En todos los casos, la presencia de cepas de *Morganella* redujeron significativamente las medidas de control de calidad comparado con el control. También, al 10^5 de unidades formadoras de colonias, la presencia de *Morganella* resultó en una mortalidad del 100% de larvas. Este estudio ilustra que *Morganella morganii* es un patógeno extremadamente letal para la mosca Mexicana de la fruta.

Palabras Clave: crianza masiva; técnica del insecto estéril; *Anastrepha*; patogenicidad

Introduction

The Mexican fruit fly, *Anastrepha ludens* (Loew) (Diptera: Tephritidae), is an economically important pest of citrus, a major pest of commercial fruit in Mexico, Central America, and South America (Enkerlin et al. 1989; Ruiz-Arce et al. 2015), and a recurrent pest in the citrus growing region in South Texas (Nilakhe et al. 1991; Conway & Forrester 2007). The larvae tunnel through the flesh of host fruits making them unmarketable. Fruit fly infestations cause serious economic losses due to yield reduction and an increase in pesticide treatment costs. Additionally, infestations can trigger

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quarantine regulations resulting in loss of markets and added fruit treatment costs for shipping.

The Mexican fruit fly is indigenous to the coastal states of Mexico (Aluja 1994; Thomas 2003) and most of Central America, as far south as Costa Rica (Flitters & Messenger 1965; Ruiz-Arce eta l. 2015). Occasional outbreaks of the Mexican fruit fly in Arizona and California starting in 1955 (Flitters & Messenger 1965; Papadopoulos et al 2013) are likely due to periodic range expansion of the fly, which is found in citrus growing regions of the coastal state of Sonora in Mexico, although the exact historical cause for the outbreaks may be uncertain. Mexican fruit flies also were captured in Sarasota, Florida, USA, in 1972 (Clark et al. 1996), and are subject to ongoing monitoring and possible eradication efforts by the United States Department of Agriculture and Florida Department of Agriculture and Consumer Service. Currently, the Mexican fruit fly has been found sporadically in all major citrus growing regions in the United States, but has yet to become established in Arizona, California, or Florida (EPPO 2017).

Due to recurring infestations in South Texas, biological control methods have been employed to control Mexican fruit fly. Sterile Insect Technique is used to suppress and eradicate Mexican fruit fly from the citrus growing areas in the Lower Rio Grande Valley in Texas (Thomas et al. 1999). For this purpose, millions of Mexican fruit flies are mass reared weekly, irradiated at the Mexican Fruit Fly Mass Rearing Facility (Moore Air Base, Mission, Texas, USA), and aerially released over citrus groves in the Lower Rio Grande Valley.

A major issue for mass rearing and production of any insect is microbial contamination (Sikorowski & Lawrence 1994; Cohen 2003; Cohen 2015). For example, Sikorowski et al. (1992) found that *Pseudomonas maltophilia* caused high mortality to the parasitoid wasp *Microplitis croceipes* (Cresson) (Braconidae: Hymenoptera). Bacterial species included in the genera *Enterobacter*, *Proteus*, and *Serratia* can also become facultative pathogens of insects (Sikorowski et al. 2001; Tanada & Kaya 1993). Although there have been no previous reports of isolation from mass rearing of Mexican fruit fly, bacteria have been isolated from laboratory and field specimens (Kuzina et al. 2001; Martinez et al. 2012).

The management of microbial contamination is a complex and difficult process, especially when rearing insects susceptible to microbial pathogens, and additionally when microbes compete for the nutrients present in the insect diet (Parker 2005; Cohen 2003; Cohen 2015). Insect mass rearing facilities require precisely controlled environmental conditions (humidity and temperature) to support rearing insects. These conditions coincide with environment conditions that favor the growth and development of microbes (Sikorowski & Lawrence 1994; Cohen 2015). Thus, adherence to strict sanitation procedures within the mass rearing facility, including sterilization of equipment and supplies, is a common necessary practice. To ensure successful insect rearing, antimicrobials are used to suppress or eliminate microbes in insect diets (Cohen 2003; Cohen 2015).

Because microbes such as bacteria are ubiquitous, identifying bacteria on the mass-rearing operation is crucial for maintaining the health of the fruit flies and viability of the colonies. There are few studies on bacteria associated with Mexican fruit fly. Rubio and McFadden (1966) reported 21 different bacteria isolated from the gut of the Mexican fruit fly collected in Mexico with *Staphylococcus* being the most frequently (76%) observed. Martinez et al. (1994) isolated 5 species of bacteria, 4 from the family Enterobacteriaceae including: *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus vulgaris*, and *Klebsiella oxytoca*, and 1 bacterium from the family Moraxellaceae: *Acinetobacter calcoaceticus* var *anitricus*, as well as several gram positive bacteria from the alimentary tract of field collected Mexican fruit fly. Kuzina et al. (2001) isolated 18 species of bacteria from the intestinal tract of the Nuevo Leon strain of mass reared Mexican fruit fly, with *Enterobacter*, *Providencia*, *Serratia*, and *Staphylococcus* as the most frequent genera, and *Citrobacter*, *Streptococcus*, *Aerococcus*, and *Listeria* less frequently found.

In 2007, at Moore Air Base in Mission, Texas, USA, we isolated microbes present in and on eggs, larvae, artificial diet, and from collected indoor air near larvae separation to establish a base line list of microbes associated with Mexican fruit fly from the rearing facility. Since then, more than 100 species of microbes have been cultured (Salas B, unpublished). In preliminary collections, isolates of *Morganella* (Enterobacteriales: Enterobacteriaceae) were associated with a "bad tray": a diet tray with low larval activity, the presence of numerous brownish or blackish dead larvae, and a wet, dark, crusty diet (Salas B & Vacek D, unpublished). The effect of bacterial contamination causing bad trays reduces viability of the mass rearing program production of 150 million Mexican fruit fly pupae per week. The bacteria can potentially contaminate the colony and may destroy fly production in the Mexican fruit fly mass rearing facility. Thus, the objective of this study was to confirm that the *Morganella* isolates found during monitoring of microbes, especially isolates obtained from morbid larvae, were pathogenic to Mexican fruit fly.

Materials and Methods

ISOLATION OF BACTERIA

In October 2010, Isolate 2232, identified as *Morganella* sp., was isolated while sampling the indoor air of the Mexican Fruit Fly Mass Rearing Facility at Moore Field in Mission, Texas, USA. At this time, the facility produced the Nuevo Leon strain of Mexican fruit fly. Isolate 2232 was obtained from bacterial colonies developing on a Petri plate with Difco™ Pseudomonas Agar F (Becton, Dickinson and Company, Sparks, Maryland, USA) amended with the antibacterial Canker Guard® (FLO TEC INC., Largo, Florida, USA). This Petri plate was attached to a SAS Super 100™ Air Sampler (Bioscience International, Rockford, Maryland, USA) and exposed for 10 s while walking in an area where Mexican fruit fly larvae were being tumbled, separated, and harvested from spent Mexican fruit fly diet.

Another *Morganella* sp. (Isolate 2431) was obtained from a live white Nuevo Leon strain larva collected aseptically from a bad tray among many dead larvae in Nov 2011. The larva was surface sterilized in 70% ethanol for 30 s then in 0.05% sodium hypochlorite (The Clorox Company, Oakland, California, USA) for 60 s, and rinsed 3 times in reverse osmosis sterile water. The larva was homogenized with a plastic pestle in an Eppendorf™ tube (Eppendorf AG 2231, Hamburg, Germany) with 1 ml of saline solution. Serial dilutions of 100 µl from 10^{-1} to 10^{-4} were prepared then spread onto Difco™ MacConkey Agar medium (Becton, Dickinson and Company, Sparks, Maryland, USA) and incubated to culture the bacteria.

All steps for the isolation of bacteria from a larva were performed within a laminar flow hood. Bacterial colonies with different morphology in shape and color were purified through repeated sub-culturing by streaking single colonies onto new plates until only 1 single colony morphology was observed. Assessment of the numbers and morphology of colonies growing on each culture plate was made after 72 h of incubation. All culture plates were incubated in darkness at 28 °C for 48 to 72 h. Isolates were stored in 15% glycerol at –80 °C to store specimens prior to DNA analysis and for future reference and use.

GENETIC IDENTIFICATION OF BACTERIAL ISOLATES

Initial genetic identification was based on sequence data from a 500 bp region of 16S rDNA that covers the variable 1, variable 2, and part of the variable 3 region of the gene (GenBank Accession Numbers SAMN06165949, SAMN06165950, Sequence GenBank Number KY364887, KY364896). Pure cultures were sent to Accugenix (Accugenix, Inc., Newark, Delaware, USA) for DNA isolation, polymerase chain reaction amplification, sequencing and identification. To more accurately identify the 2 cultures, an additional 5 genes (ATP synthase subunit B [*atpD*], DNA polymerase III subunit B [*dnaN*], DNA gyrase subunit B [*gyrB*], translation initiation factor IF-2 [*infB*], and RNA polymerase subunit B [*rpoB*] were sequenced by Erin Schuenzel using the methods of Emborg et al. (2006) (Table 1) and compared to the 22 sequences known for *M. morganii* and *M. psychrotolerans* from Emborg et al. (2006). Gene sequences from this study were submitted to GenBank (Accession Numbers SAMN06165949, SAMN06165950, Sequence Number KY364887, KY364896). Finally, the whole genome shotguns contigs of the 2 isolates were sequenced and assembled.

DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA). For gene amplification and sequencing, protocols in Emborg et al. (2006) were followed. Amplified products were cleaned with Exo-Sap-It (Affymetrix, Santa Clara, California, USA) and sent to the University of Chicago Cancer Center DNA Sequencing and Genotyping Facility (Chicago, Illinois, USA) for sequencing on an ABI 3730 using BigDye Terminator (ABI, ThermoFisher, Waltham, Massachusetts, USA). The same extracted DNA was used for whole genome sequencing. The Nextera® XT DNA Library Preparation Kit (Illumina, San Diego, California, USA) and Index Kit (Illumina, San Diego, California, USA) were used to create sequencing libraries for both isolates. The genomes were sequenced on an Illumina MiSeq personal sequencer using the Nano Kit v2 (500 cycles) (Illumina, San Diego, California, USA).

The sequences for the 5 genes were edited and aligned to known sequences using BioEdit v 7.2.5 (Hall 1999). Genome sequencing reads were assembled using Velvet v1.2.10 (Zerbino & Birney 2008, Zerbino et al. 2009) and compared to the reference genome *M. morganii* subsp. *morganii* strain KT (NC_020418) using Geneious v 7.1.7 (Biomatters Ltd.).

PATHOGENICITY TESTS

Bioassays on Mexican fruit flies were conducted from Nov 2011 to Feb 2014 with analysis of the *Morganella* data running from Nov 2011 to Sep 2012. The adult flies fed on a diet of hydrolyzed yeast and sugar designed to maximize adult longevity and egg production (Martinez et al. 1987). Eggs collected from the egg laying panels at Center for Plant Health Science and Technology Mex Fly Methods Development Laboratory were washed 4 times in reverse osmosis water. Subsequently, 50 to 100 ml (average 20,000 eggs per ml) were bubbled in a 2 L flask using a fish aerator for 4 d in a mixture of 2,000 ml of reverse osmosis water amended with 24 ml of 360 ppm H_2O_2 (Aaron Industries, Clinton, South Carolina, USA), and 2 ml of 1% oxolinic acid (Sigma-Aldrich, St. Louis, Missouri, USA). Eggs were strained and suspended at a 1:9 ratio (eggs to agar) in agar mix. Agar mix was prepared with 1.89 L of H_2O, amended with 3.5 g methyl paraben (Aakash Chemicals, Glendale Heights, Illinois, USA), and 2.625 g of agar (Marcor Development Corporation, Carlstadt, New Jersey, USA). A total of 0.4 ml of egg agar suspension (about 600 eggs) was pipetted onto 50 g of freshly prepared Mexican fruit fly meridic diet inside a 198 g capacity polypropylene plastic cup (Highland Plastics, Pasadena, California, USA).

The artificial diet used to feed larvae was modified from Spishakoff and Hernandez-Davila (1968). A modified meridic diet formula (Conway H, unpublished) was prepared by mixing ingredients listed in Table 2 into a 60 kg stainless steel bowl by pouring 19 L of water, Bravo Weather Stick® (Syngenta Crop Production LLC, Greensboro, North Carolina, USA), and hydrochloric acid followed by all of the solid ingredients and the remaining water. Diet ingredients were mixed in a 60 qt Hobart mixer for 15 m. Each batch of diet was tested and adjusted to pH 3.75 ± 0.25 to inhibit microbial growth. Aliquots of 50 g each were taken from the final diet mix and used in the small diet cup pathogenicity testing.

Pathogenicity was defined as reduction in the number of viable insects as indicated by weight differences per cup. Under small scale rearing conditions, weight differences were due to smaller size and mortality caused by the tested bacteria to eggs, instars, larvae, and pupae compared to normal treatment controls. For pathogenicity tests, *Morganella* isolates were grown on BBL™ Trypticase™ Soy Agar (Becton, Dickinson and Company, Sparks, Maryland, USA) for 48 h at 28 °C. Inoculum was prepared by transferring a few bacterial colonies into serial dilution tubes which were adjusted to the desired concentration with the aid of a spectrophotometer. Initially, 3 concentrations (10^5 colony forming units [CFU] per ml, 10^6 CFU per ml, and 10^7 CFU per ml) of *Morganella* were tested in bioassays against fruit fly larvae using 2 inoculation methods, immersion and surface. Because the 2 highest concentrations were found to be extremely lethal to Mexican fruit fly eggs, 2 lower concentrations (10^3 CFU per ml, and 10^2 CFU per ml) were added later to compare to 10^5 CFU per ml.

The immersion method consisted of mixing the egg suspension with 1 ml of *Morganella* suspension, incubating for 1 h at room temperature (25–26 °C), and pouring the mixture onto 50 g of Mexican fruit fly meridic diet in a 198 g capacity polypropylene plastic bioassay cup (Highland Plastics, Pasadena, California, USA). The surface method consisted of infesting the diet with 0.4 ml of egg agar suspension (about 600 eggs) 1 to 2 h prior to bacterial inoculation by spreading the diet surface with 1 ml of *Morganella* suspension. Control cups were established by adding 1 ml of sterile water. After inoculation, bioassay cups were covered with propylene lids to maintain a high humidity and temperature. After 72 h, propylene lids were replaced with screened lids (1.5 × 1.5 mm grid) to allow oxygen flow and remove excess heat from the rapidly growing larvae. This methodology resulted in a factorial experimental design for each *Morganella* isolate (2 infestation methods × 4 inoculum levels) plus control in a completely randomized design with 3 reps per treatment.

Table 1. Primers and annealing temperature conditions for 5 MLST genes derived from Emborg et al. 2006

Gene target	Sequence (5′→3′)	Annealing temperature (°C)
atpD	GGAAGTTCAGCAGCAGTTAG TATCAACGAACAGCAGTACG	51
dnaN	ATGAAATTTACCGTTGAACGTGA CCATCCACCAGCTTCGAGGT	51
gyrB	ATAAGTTCGACGATAACTCC TGGTCTGAGAGGAGAATTTC	51
infB	TTACTGGACTATATCCGTTC ATATCGCCTTTGTTCAGTG	49
rpoB	CTTTGGTTCCAGCCAGCTG TCAATGCACGGTTGGCGTC	58

Table 2. Meridic Mexican fruit fly diet used for small diet cup tests.

Ingredients	Source	Metric amount	Percent of diet (%)
Corn Cob M80	The Andersons	8 kg	15.74
Sugar	United Sugar Corp	4.2 kg	8.27
Torula Yeast	Great Lakes	3.2 kg	6.29
Wheat Germ	North Dakota Mill	2.4 kg	4.72
Toasted Soy	ADM	1.8 kg	3.54
Methyl Paraben	Aakash Chemicals	0.115 kg	0.22
Sodium Benzoate	Westco Chemical Inc.	0.05 kg	0.10
Bravo WS (1% sol.)	Syngenta	0.05 ml	0.10
Hydrochloric Acid	Valley Solvents & Chemical	0.50 L	1.00
Water		30.5 L	60.02
Totals		50.815	100

All pathogenicity tests were conducted in a growth chamber at the CPHST Arthropod Quarantine Facility in Mission, Texas, USA, set to 26.5 ± 1.5 °C, 82.5 ± 2.5% RH, and 14:10 h L:D photoperiod. To avoid cross contamination, small observation cages were labeled and randomized on racks. Each cage received 3 diet cups (reps) with the same treatment. Separation of larvae from the diet was performed after 9 to 10 d post inoculation. If diet in the bioassay cup was extremely wet and sticky, larvae were carefully separated from diet by washing through a 1.5 × 1.5 mm grid hand held strainer (Farberware®, Fairfield, California, USA) with tap water. When the diet was dry and powdery, larvae were separated by sifting with a 1.5 × 1.5 mm grid hand held strainer. Larval weights were taken within 2 h of washing or sifting. The sifted larvae were placed into a new, labeled bioassay cup, containing corn cob grit to enhance pupation.

PUPAL WEIGHT, ADULT EMERGENCE, AND FLIGHT ABILITY

Mean pupa weight, percent adult emergence, and percent flight ability were determined using the quality control standard testing procedures described in FAO-IAEA-USDA Manual for Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies (2003). Pupae weight was obtained after 21 d post egg infestation, which is approximately 2 d prior to expected adult emergence. If available, up to 100 pupae per cup were counted and analyzed to obtain average pupa mass.

For percent adult emergence and flight ability, up to 100 pupae were placed within a paper ring inside a black plastic flight ability tube (11 cm tall × 8 cm diam) with talc applied to the interior surface. The paper ring was centered in the bottom of a Petri dish positioned on the bottom of the flight ability tube. After approximately 5 d, when all flies had emerged and died inside the observation cage, the percent emergence was calculated. The percent flight ability was calculated by taking the value for percent emergence and subtracting the number of deformed flies and non-fliers found inside the flight ability tube. Survivorship from egg to pupae was calculated based on the number of pupae produced per cup divided by the estimated number of starting eggs.

STATISTICAL ANALYSIS

Larval weight, pupae mass, pupae per cup, percent adult emergence, and percent flight ability data were analyzed by ANOVA with Tukey's HSD with all pairwise comparisons test of means at $P = 0.05$ using JMP 10 (SAS Institute, Cary, North Carolina, USA) (2003). Because *Morganella* isolates were lethal at concentrations of 10^6 and 10^7 (mortality of about 100%), these concentrations were not included in the statistical analysis

Results

GENETIC IDENTIFICATION

Both isolates 2232 and 2431 were putatively identified as belonging to the genus *Morganella*. While the 16S rRNA locus is the standard for bacterial species identification, it is only 1 gene and only reliably iden-

Table 3. Larval weights and activity of Mexican fruit fly larvae treated with varying concentrations of 2 *Morganella morganii* isolates.

	Larval mass yield ± SE (g)		Estimated reduced weight (%)[1]	Behavior at 72 h post-inoculation[2]	
Morganella isolates and CFU concentrations	Dead	Live		Motionless	Vigorous
Isolate 2431					
10^2 CFU per ml	0.44 ± 0.14	3.10 ± 0.84	77.08	0.76	29.13
10^3 CFU per ml	0.06 ± 0.01	0.43 ± 0.15	96.84	3.43	14.30
10^5 CFU per ml	0.0	0.0	100.00	17.76	0.02
Isolate 2232					
10^2 CFU per ml	0.90 ± 0.24	3.88 ± 1.01	71.27	0.69	33.20
10^3 CFU per ml	0.75 ± 0.36	1.57 ± 0.48	88.40	1.67	22.80
10^5 CFU per ml	0.01 ± 0.00	0.01 ± 0.01	99.92	11.46	0.59
Control					
0 CFU per ml	0.02 ± 0.01	13.51 ±0.24		0.17	40.19

[1]Percent loss calculated by: 100-[(treatment yield/control yield)*100].
[2]Counts under stereo microscope field: 10× by 0.63×.

tifies to the genus level. The multiple genes sequenced (ATP synthase subunit B [*atpD*], DNA polymerase III subunit B [*dnaN*], DNA gyrase subunit B [*gyrB*], translation initiation factor IF-2 [*infB*], and RNA polymerase subunit B [*rpoB*]) give a more robust answer to the taxonomic identity of the 2 highly pathogenic isolates. With regard to the 5 genes, isolate 2232 shared an average percent similarity with the 9 known *M. morganii* strains of 96.7% and an average percent similarity with the 13 known *M. psychrotolerans* strains of 88.0% (the nearest known taxa) using a pairwise similarity matrix produced by BioEdit (Hall 1999). The genome sequencing of this isolate generated 410 contigs totaling 3.8 Mb. When compared to known annotated genome sequences using the non-redundant nucleotide megaBLAST (Geneious 7.1.7) (Biomatters Limited, Auckland, New Zealand), the *M. morganii* subsp. *morganii* strain KT (NC_020418) was the only subject returned against the consensus sequence constructed with the contigs for isolate 2232.

Isolate 2431 was only identified to the genus level using the 16S rRNA locus. The concatenated dataset of 5 genes revealed a 96.7% similarity with *M. morganii* and an 88.1% similarity with *M. psychrotolerans.* The genome sequencing provided more data to distinguish the isolate taxonomically. Sequencing provided 628 contigs totaling 3.8 Mb. Again, *M. morganii* subsp. *morganii* strain KT (NC_020418) was the only subject returned by megaBLAST for isolate 2431. All the sequence data indicates that these 2 isolates, 2232 and 2431, are *M. morganii.*

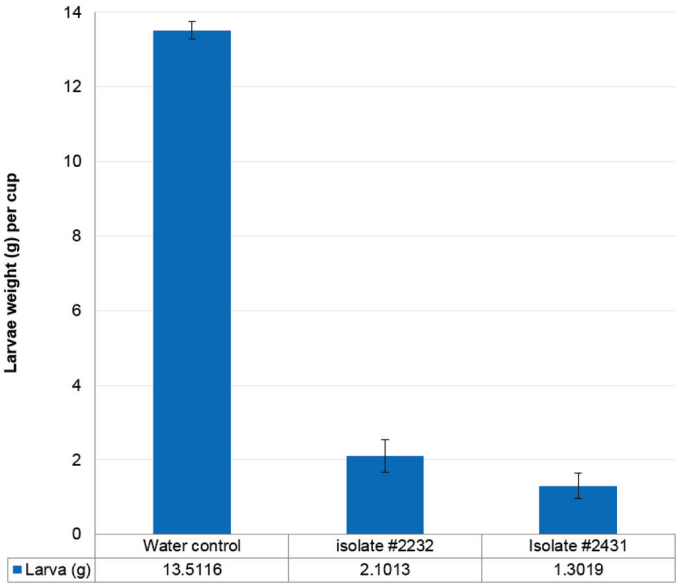

Fig. 1. Mean weight ± SE of Mexican fruit fly larvae produced per cup (g).

PATHOGENICITY TESTS

Larval Weight

There was no significant difference between the 2 methods of inoculation of Isolate 2431 and Isolate 2232 for larval mass per cup (± SE), with egg emersion 0.86 ± 0.21 g and surface inoculation 0.84 ± 0.22 g ($F_{1,215}$ = 0.002; P > 0.97), pupa production (± SE) with egg emersion 11.2 ± 2.6 and surface inoculation 10.9 ± 2.8 pupae per cup ($F_{1,215}$ = 0.006; P > 0.93), percent adult emergence (± SE) with egg emersion 7.1 ± 1.3% and surface inoculation 6.4 ± 1.2% ($F_{1,205}$ = 0.149; P > 0.71), or percent flight ability (± SE) with egg emersion 6.3 ± 1.1% and surface inoculation 6.3 ± 1.2% ($F_{1,205}$ = 0.0003; P > 0.99). The direct deleterious effect on larvae of Mexican fruit fly by both isolates of *Morganella* was evident after 72 h of inoculations. Larvae in bioassay cups examined under stereo microscope either were moribund or actively moving (vigorous). Counts of motionless larvae (presumed dead), increased as inoculum levels increased (Table 3). In contrast, counts of vigorous larvae decreased with an increase of inoculum concentrations. Larval yield of the 2 *Morganella* isolates was significantly affected by inoculum concentration for larval mass ± SE of 3.85 ± 0.61ml for 10^2 CFU per ml, 1.25 ± 0.37 ml for 10^3 CFU per ml, and 0.006 ± 0.004 ml for 10^5 CFU per ml ($F_{2,116}$ = 27.153; P > 0.0001). Concentrations equal to 10^5 CFU per ml killed 99 to 100% of larvae. Concentrations of 10^3 CFU per ml of isolate 2431 and 2232 produced less than 0.426 and 1.567 g of larvae, respectively (Table 3). Isolate 2431 reduced live larval yield by 77%, 97% and 100% with inoculum concentrations of 10^2, 10^3, and 10^5, respectively (Table 3). Similarly, Isolate 2232 reduced live larval yield loss by 71%, 88% and 99.9% with inoculum concentrations of 10^2, 10^3, and 10^5, respectively (Table 3). The control cups had significantly greater larvae yield ($F_{2, 203}$ = 316.119, P < 0.0001) than Isolate 2431 and Isolate 2232 (Fig. 1).

Before sifting, bioassay cups inoculated with *Morganella* isolates produced hard, wet, sticky, and non-processed diet (Fig. 2) that required washing instead of sifting for extracting live larvae from diet. The few surviving larvae from *Morganella* treatments were smaller in size and weight than larvae in control bioassay cups (Fig. 3). Surviving larvae exposed to *Morganella* were often brownish or blackish in color. In contrast, larger and healthy whitish larvae were present in control cups (Fig. 3).

Pupa Weight, Adult Emergence, and Flight Ability

The mean weight (± SE) of individual pupae from control cups was significantly greater (19.3 ± 0.3 mg) than the mean from *Morganella*-treated cups (16.2 ± 2.3 mg), ($F_{2, 82}$ = 6.504, P > 0.002). The control cups contained a significantly greater number of pupae per 50 g of diet than the cups infested with either of the *Morganella* isolates (Fig. 4). The control cups contained 73× greater pupal yield than Isolate 2232 and 155× more pupal yield than Isolate 2431 ($F_{2,203}$ = 1845.296, P < 0.0001) (Fig. 4). Conversion from eggs to pupae for the control, Isolate 2232, and Isolate 2431 was 72.10%, 0.99%, and 0.47%, respectively. Less than 1% of the starting eggs emerged as larvae and formed pupae when in the presence of either *Morganella* isolate.

The percent adult emergence ($F_{2,82}$ = 130.256, P < 0.0001) and percent flight ability of adult flies ($F_{2,82}$ = 143.652, P < 0.0001) from the control was significantly higher than values of flies recorded for either of the *Morganella* isolates (Fig. 5). All adult flies from the *Morganella* treatments exhibited undesirable low mean % emergence (± SE) and reduced flight ability of around 25 ± 5% compared to 81 ± 1% in the controls.

Overall, Mexican fruit fly production was negatively impacted ($F_{2, 203}$ = 6606.179, P< 0.0001) by the presence of either of the *Morganella* isolates (Fig. 6). Conversion from egg to adult fly for the control, Isolate 2232, and Isolate 2431 was 58.30%, 0.22%, and 0.12%, respectively. Less than 1% of the starting eggs survived to adult stage when in the presence of either *Morganella* isolate.

Control methods have since been developed for bacteria including *Morganella*, consisting of improved sanitary methods for equipment and the use of rinses of Mexican fruit fly eggs with 150 ppm Clorox® bleach across 3 d of egg incubation and 1 rinse of 50 ppm Betadine® Microbicides (Purdue products L.P., Stamford, Connecticut, USA) solution prior to mixing with agar and infesting on top of diet (Salas B, Leal S, Thomas D & Conway H, unpublished). An additional control method that has been incorporated in the rearing process is sampling of new shipment of bulk diet ingredients for microbial contamination. This ensures relatively clean starting diet ingredients in the artificial diet mix.

Fig. 2. Bio-assay diet cups at sifting: Left=Control, Right=inoculated with *Morganella morganii*.

Discussion

Details of the taxonomy and binomial changes over time of *Morganella* can be found in O'Hara et al. (2000). Briefly, *Morganella* was proposed by Fulton (1943), and was established as a genus within the *Enterobacteriaceae* based on genetic studies by Brenner et al. (1978). Thus, strains previously known as *Proteus morganii* became the genus *Morganella* and named *Morganella morganii* (Fulton 1943). At present, *M*. *morganii* includes 2 subspecies, *M*. *morganii* subsp. *morganii* and *M*. *morganii* subsp. s*ibonii*, which can be distinguished from each other on the basis of trehalose fermentation test (Jensen et al. 1992).

In humans, *M*. *morganii* may produce urinary tract infections, septicemia, wound infections, and travelers' diarrhea (Janda et al. 1996). In insects, *M*. *morganii* was found in the intestines of house fly larvae (*Musca domestica* [Diptera: Muscidae]) (Zurek et al. 2000), gastrointestinal tracts of *Drosophila melanogaster* (Diptera: Drosophilidae) (Cox & Gilmore 2007), adults and eggs of horn fly (*Haematobia irritans* [Diptera: Muscidae]) (Palavesam et al. 2012), midgut of phlebotomine sand fly (*Lutzomyia longipalpis* [Diptera: Psychodidae]) (Gouveia et al. 2008; Peterkova-Koci et al. 2012), gastrointestinal tracts of the Mediterranean fruit fly (*Ceratitis capitata* [Diptera: Tephritidae]) (Ami et al. 2009; Yuval et al. 2013), in isolations made from the whole body of the guava fruit fly (*Anastrepha striata* [Diptera: Tephritidae]) (Martinez et al. 2012), and in the gastrointestinal tracts of the oriental fruit fly (*Bactrocera dorsalis* [Diptera: Tephritidae]) (Pramanik et al. 2014; Liu et al. 2016). To our knowledge, no pathogenicity tests were conducted in any of these studies on fruit fly species, especially in regards to fruit fly larvae. Association of bacterial populations with Tephritidae may play a significant role in insect nutrition with the gut microbiota being relatively conserved in terms of species composition and comprised mainly of Enterobacteriaceae (Lauzon 2003).

In the past, *Morganella* was not listed among the bacteria isolated from the intestinal tracts of adult flies of *A*. *ludens* collected in 1999 from the Mexican Fruit Fly Rearing Facility in Mission Texas (Kuzina et al. 2001). It is impossible to ascertain if *Morganella* was among the 16 bacterial types reported in Rubio & McFadden (1966); consequently, to our knowledge, this is the first report of a *Morganella* species that is pathogenic to Mexican fruit fly. All previous isolations were made from adult flies (Rubio & McFadden 1966; Martinez et al. 1994; Kuzina et al. 2001; Yuval et al. 2013; Pramanik et al. 2014; Liu et al. 2016). In our studies, Isolate 2431 was isolated from a homogenized white larva which was among the brownish and blackish collected from a bad tray, and Isolate 2232 was obtained while sampling (SAS Super 100™ Air Sampler Bioscience International, Rockford, Maryland) the indoor air where Mexican fruit fly larvae were being separated from diet at the Mexican Fruit Fly Mass Rearing Facility in Mission, Texas.

We have found bacteria from the families Enterobacteriaceae, Bacillaceae, Pseudomonadaceae, and others while sampling at the Mexican fruit fly mass rearing facility. The effect of bacteria associated with the mass rearing of Mexican fruit fly has not previously been examined in detail. In this study, we have demonstrated that *Morganella morganii* is extremely detrimental to eggs and larvae of *A*. *ludens.* The presence of *Morganella* can result in 100% larval yield loss especially when *Morganella* is present in high concentrations. The few surviving

Fig. 3. Yield of Mexican fruit fly larvae in bio-assay cups. Left=Control, Right=Inoculated with *Morganella morganii*.

adult flies from the *Morganella* treatments were smaller in size and had undesirable low emergence and flight ability values compared to the larger, more numerous flies from the control treatment. Thus, the presence of *Morganella* in mass rearing facilities negatively affects the suppression and eradication effort of the Mexican fruit fly because of the resultant reduction in the number of sterile flies available for aerial release.

Finally, *Morganella*-inoculated diet cups display physical characteristic seen in the bad trays, i.e., wet, sticky, and dark diet with a foul smell containing brownish or blackish larvae. We propose that *Morganella* can cause the bad trays presence during the mass rearing of Mexican fruit fly at the Mexican Fruit Fly Mass Rearing Facility in Edinburg, Texas, USA. Both the Mexican fruit fly strain from Tapachula, Mexico that was introduced and used from 2001 to 2009 and the Nuevo Leon strain used from 2009 to 2012 displayed the bad tray syndrome. This bad tray syndrome often results in a 10 to 15% production loss, which correlates to a reduction of 6 to 12 million Mexican fruit flies per week. Interestingly, the Willacy strain of Mexican fruit fly, which was introduced in Dec 2012, does not exhibit the bad tray syndrome. With Willacy strain, occasionally there are larval trays with black rings of death (necrotic dead black larvae surrounded by discolored diet) ranging from 2 to 20 cm in diameter (Fig. 7). The dead black larvae seem to originate from a single point source extending outward as well as vertically in the tray. *Morganella* likewise has been isolated from these dead black larvae (Salas B & Vacek D, unpublished). Furthermore, a single, subsurface, point inoculation of 10 ml of 10^5 CFU per ml of *Morganella* sp. into the edge of the diet in bioassay cups causes 100% mortality (Salas B & Vacek D, unpublished). This implies that a tiny, point contamination of diet by *Morganella* could cause the dark rings of death.

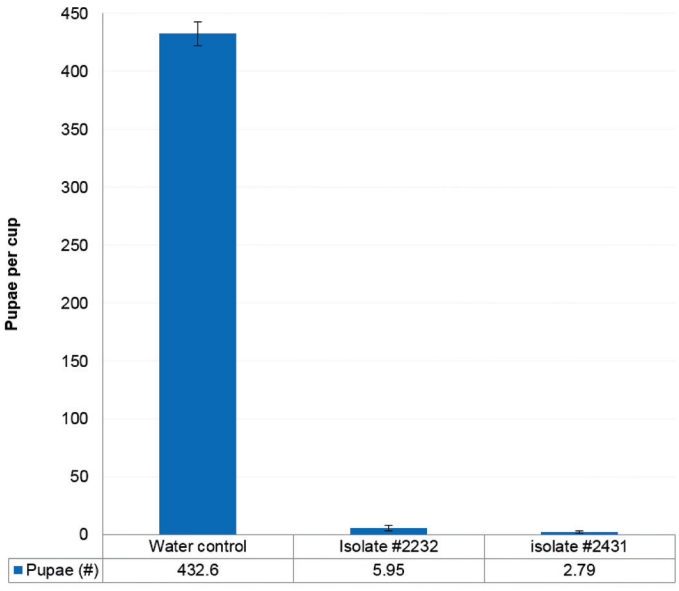

Fig. 4. Mean number ± SE of Mexican fruit fly pupae produced per cup (#).

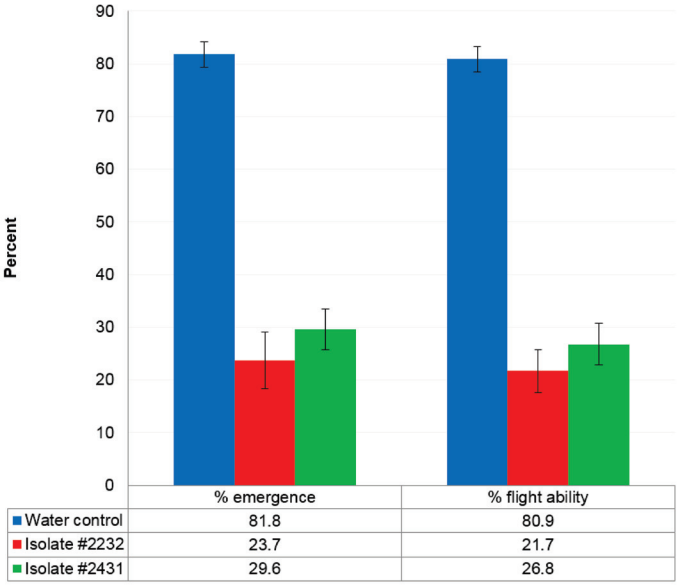

Fig. 5. Percent adult emergence ± SE and percent flight ability ± SE by treatment type.

Only 1 other study has found a *Morganella* isolate pathogenic to insects: the larvae of phlebotomine sand flies *L. longipalpis* (Lutz and Neiva) (Peterkova-Koci et al. 2012). Unfortunately, previous researchers did not report if *Morganella* was pathogenic or non-pathogenic when found.

Acknowledgements

We would like to thank Mateo Hernandez, Juan Veliz, Gilberto Salinas, and Armando Loya, United States Department of Agriculture, Animal Plant Health Inspection Service, Plant Protection Quarantine, Center for Plant Health Science Technology, and Roxanne Ramos, Emily Sanchez, Johnathon Waggoner, Kassandra Cantu, and Mark Scally, University of Texas Rio Grande Valley, for assisting with this study. We also thank Evan Braswell and Norman Barr (USDA APHIS PPQ CPHST Mission Laboratory) for their critical review of the draft of this manuscript and providing valuable comments. Additionally, we thank Sandra Leal (USDA APHIS PPQ Harlingen, Texas) and Sylvia Barr for assistance with the Spanish Resumen. Funding for the research supported by U.S. Department of Agriculture-Animal Plant Health Inspection Services award number 12-8130-0042-CA to C. Vitek, and by USDA HSI Education Program award number 2011-38422-30826 USDA, and NSF Expanding the Network of STEM Scholars award number 1463991 to E. Schuenzel.

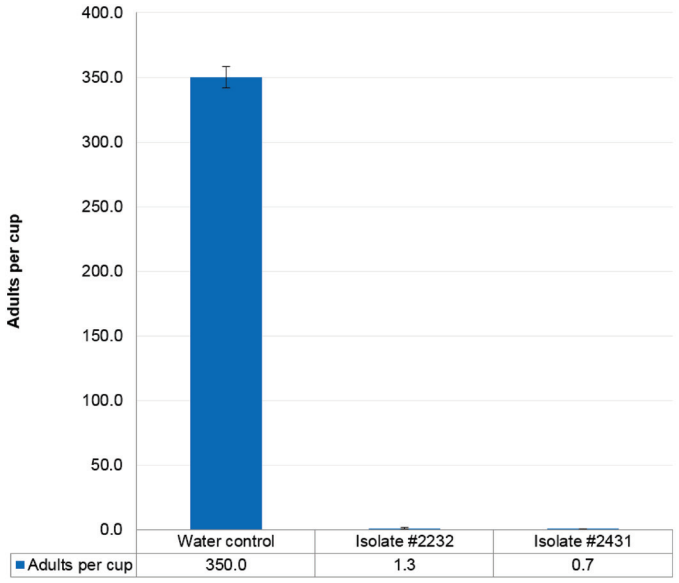

Fig. 6. Adult Mexican fruit fly ± SE produced from 50 g of diet.

Fig. 7. Black ring of death around dead Mexican fruit fly larvae in diet.

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