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IDENTIFICACIÓN Y ANÁLISIS DE LAS BACTERIAS SIMBIONTES DEL GÉNERO *Dactylopius* (HEMIPTERA: DACTYLOPIIDAE)

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P R E S E N T A

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RESUMEN

Los insectos del género *Dactylopius* (Dactylopiidae), conocidos como cochinilla del nopal o grana cochinilla, son parásitos que se alimentan únicamente de la savia de los nopales. El objetivo de este estudio fue determinar cuáles son las bacterias simbiontes de los insectos del género *Dactylopius* (Dactylopiidae).

Se realizó un análisis de la diversidad bacteriana mediante un enfoque independiente de cultivo utilizando secuencias del gen 16S rRNA que se obtuvieron por PCR de DNA metagenómico aislado de distintas especies de *Dactylopius* colectadas en México y Brasil. Se encontraron bacterias de distintos géneros como *Massilia, Herbaspirillum, Acinetobacter, Mesorhizobium, Sphingomonas*, *Candidatus* Hepatincola porcellionum y una betaproteobacteria. No todas las muestras tuvieron las mismas bacterias, sólo la betaproteobacteria fue común en la mayoría de las muestras.

Mediante la secuenciación del metagenoma de *Dactylopius coccus* se encontraron en una gran proporción secuencias de *Wolbachia* de los supergrupos A y B, siendo mayoritario el B. Se logró ensamblar parcialmente sus genomas. La evidencia sugiere que existe una infección múltiple por dos cepas de *Wolbachia* en algunos individuos. Además, mediante análisis filogenómicos, de identidad de nucleótidos (ANI) y de hibridación DNA-DNA (DDH) *in silico,* se analizaron las relaciones evolutivas de cepas de *Wolbachia* de diferentes supergrupos mostrando que pudieran corresponder a distintas especies.

ABSTRACT

Insects from genus *Dactylopius* (Dactylopiidae), known as cochineals, are parasites that feed only by cactus sap. The goal of this study was determine the symbiotic bacteria of insects from genus *Dactylopius* (Dactylopiidae).

Bacteria diversity analysis was performed through a culture-independent approach using 16S rRNA gene sequences obtained from PCR from metagenomic DNA isolated from different *Dactylopius* species collected in Mexico and Brazil. Bacteria from different genera like *Massilia, Herbaspirillum, Acinetobacter, Mesorhizobium, Sphingomonas*, *Candidatus* Hepatincola porcellionum, were found and a betaproteobacteria. No all the cochineal species shared the same bacteria, only the betaproteobacteria was present in most of the samples.

Through metagenome sequencing of *D. coccus,* we found sequences of *Wolbachia* from supergroups A and B in a high proportion, being the majority of supergroup B. Genomes of the two strains were partially assembled and evidence suggests that a multiple infection by two *Wolbachia* strains exists in some individuals. Further, by phylogenomic analyses, Average nucleotide identity (ANI), and in silico DNA-DNA hybridization, we analyzed the evolutionary relationships of *Wolbachia* strains from different supergroups showing that they may belong to different species.

INTRODUCCIÓN

Definición de simbiosis

En 1873, el zoólogo belga Pierre-Joseph van Beneden propuso los términos "parasitismo", "comensalismo" y "mutualismo" para referirse a los diferentes tipos de relaciones de "animales inferiores" que vivían dentro de "animales superiores" (Sapp, 1994), con la intención de enfatizar que no todas las relaciones entre animales "inferiores" y "superiores" son de naturaleza parasitaria. En 1877, Albert Bernhard Frank acuñó el término "simbiotismo" (*symbiotismus*) con la intención de agrupar en un mismo concepto todos los casos en los que dos especies diferentes viven "sobre o en otra", basándose en su mera coexistencia. En 1878, Anton de Bary usó por primera vez el término "simbiosis" en el ensayo "The Phenomena of Symbiosis" expuesto durante la reunión general de la Asociación de Físicos y naturalistas alemanes realizada en Cassel en ese año. Bajo este concepto incluyó varios tipos de asociaciones complejas que se encontraban en un rango continuo desde parasíticas hasta mutualistas (Sapp, 1994).

Actualmente, simbiosis se define como cualquier relación que se establece entre dos o más individuos filogenéticamente distintos, es decir, de diferentes especies, sin importar el efecto que tienen unos en los otros. Los participantes de dichas asociaciones se conocen como simbionte y hospedero siendo este último el organismo que alberga al primero. De acuerdo al efecto del simbionte en su hospedero se divide en tres tipos:

Simbiosis parasitaria: es aquella en la que una de las especies involucradas vive a expensas de la otra causándole daño.

Simbiosis comensalista: es aquella en la que una de las especies obtiene un beneficio de la otra sin tener efectos negativos en ella.

Simbiosis mutualista: es aquella en la que las especies involucradas reciben beneficios como resultado de su asociación.

Uno de los aspectos más significativos de la simbiosis es que conduce a variabilidad en los individuos de una población que a su vez resulta en novedades evolutivas en adición a la evolución gradual por Selección natural sobre la acumulación de variaciones individuales (Moran, 2006; Sapp, 1994). Se ha propuesto que las simbiosis mutualistas entre organismos multicelulares y unicelulares han contribuido significativamente a la evolución de la vida en la Tierra y quizás el ejemplo más claro que tenemos es el surgmiento de la célula eucarionte y los organelos como cloroplastos y mitocondrias a partir de cianobacterias y alfa-proteobacterias, respectivamente (moran, 2006).

Simbiosis mutualistas entre bacterias y animales

Las interacciones simbióticas entre bacterias y animales se encuentran ampliamente distribuidas en la naturaleza, de hecho, en condiciones naturales los eucariontes no están libres de bacterias u otros microorganismos por lo que el funcionamiento de su metabolismo no se puede concebir sin la influencia de su microbiota.

Las simbiosis más estudiadas han sido las parasitarias en un esfuerzo por contrarrestar los efectos que causan en el ser humano, sin embargo, existe un creciente interés en el estudio de las relaciones mutualistas ya que establecer simbiosis de este tipo le permite a los eucariontes obtener recursos a los cuales no tienen acceso (Moran, 2006). Se ha propuesto que el éxito de los eucariontes para colonizar diferentes ambientes se debe a su antigua y continua capacidad de asociarse con bacterias (Douglas, 2012). Además, se ha demostrado que las bacterias son fundamentales para el desarrollo adecuado y la supervivencia de los animales (Douglas, 1998). Por ejemplo, el intestino humano está colonizado por 100 millones de millones de microorganismos (10^{14}) (Ley et al., 2006) que son más células de las que conforman nuestro propio cuerpo (3.72^{13}) (Bianconi et al., 2013). Hasta hace poco eran consideradas comensalistas, pero ahora se sabe que intervienen en la estimulación y desarrollo del sistema inmune (Fraune & Bosch, 2010); también influyen en el desarrollo y funcionamiento del sistema nervioso, el concepto de "eje microbioma-intestino-cerebro" supone que alteraciones en la composición microbiana del intestino están relacionadas con cambios marcados en el estado de ánimo, dolor, cognición y desórdenes asociados a estrés, ansiedad y depresión (Furness et al., 2014). Se ha observado que ratones colonizados con una microbiota normal muestran una respuesta a la ansiedad y control motor normal en comparación con ratones libres de microorganismos (Diaz Heijtz et al., 2011). Otros estudios han

sugerido que las respuestas individuales a ciertos fármacos, enfermedades como la obesidad, el síndrome de fatiga crónica y alergias a ciertos alimentos podrían ser explicados por las diferencias en la actividad de la microbiota de cada individuo (Douglas-Escobar et al., 2010)

Del mismo modo, en ambientes marinos muchos animales poseen simbiontes bacterianos. Los gusanos marinos del grupo de los pogonóforos como *Riftia pachyptila*, que viven a 2,400 metros de profundidad, obtienen sus nutrientes gracias a sus simbiontes quimiosintéticos (Dubilier et al., 2008). En la asociación de los calamares *Euprymna scolopes* y las bacterias *Vibrio fischeri,* el hospedero utiliza la luz producida por estas bacterias luminiscentes para confundir a sus depredadores durante la noche (Nyholm & McFall-Ngai, 2004). Las esponjas marinas son organismos capaces de albergar una diversa comunidad de simbiontes bacterianos que llegan a constituir hasta el 35% de su biomasa. Mediante estudios genómicos se ha propuesto que los simbiontes proveen vitaminas y otros metabolitos que sirven de defensa contra patógenos (Hentschel et al., 2012).

La simbiosis es un proceso interactivo que puede delinear la evolución de simbiontes y hospederos. En muchas asociaciones mutualistas existe una dependencia recíproca por lo que no pueden vivir separados. Actualmente, muchos eucariontes son reconocidos como una colección de células del hospedero y de los simbiontes, redefiniendo la noción de "individuo" y llevándonos más allá en nuestro entendimiento de las especies y de la vida misma (Russell et al., 2014).

Simbiosis entre bacterias e insectos

Las bacterias son el grupo de organismos más abundante y el desarrollo de nuevas técnicas de análisis ha revelado que son más diversas de lo que hubiéramos imaginado. Presentan una inmensa diversidad metabólica y se dice que son los catalizadores primarios de nutrientes en la naturaleza, lo que les permite estar distribuidos en toda la Tierra. La clasificación de bacterias se basa mayormente en información fenotípica y genética y está restringida a cepas cultivables. Solamente ~11,000 especies de bacterias y arqueas han sido clasificadas a la fecha y anualmente surgen ~600 descripciones nuevas (Yarza et al., 2014), pero cada año se reportan cientos de miles de secuencias nuevas y los estimados del número total de especies de procariontes, incluyendo bacterias y arqueas, varía entre $3x10^4$ a 10^{12} (Yarza et al., 2014).

Los insectos son el grupo de animales más diverso y exitoso sobre la Tierra dado que son capaces de establecerse en casi cualquier hábitat ya sea terrestre, de agua dulce o marino. No se sabe con exactitud cuántas especies existen, pero se calcula que hay entre 890 mil y un millón descritas y que falta por describir más de 3 millones (Brusca & Brusca, 2003). Debido a que muchos de ellos tienen dietas pobres o con compuestos difíciles de degradar es común que establezcan simbiosis con bacterias que compensan sus carencias nutricionales proveyéndoles vitaminas, aminoácidos o compuestos bioactivos (Moran, 2006). Estos simbiontes influyen en su nutrición (McCutcheon et al., 2009a), desarrollo (Tsuchida et al., 2010), reproducción (Simon et al., 2011), respuesta inmune (Weiss et al., 2012), resistencia a estrés ambiental (Burke et al., 2010), e incluso su preferencia por hábitat y alimento (Tsuchida et al., 2004). Por ejemplo, *Buchnera aphidicola*, el simbionte del áfido *Acyrthosiphon pisum*, sintetiza riboflavina y aminoácidos que después el insecto aprovecha ya que estos nutrientes son escasos en la savia de la que se alimenta (Nakabachi

& Ishikawa, 1999). Los simbiontes también sintetizan compuestos bioactivos que sus hospederos utilizan para obtener su alimento o defenderse de depredadores (Moran, 2007), como en el caso de las termitas en las que las bacterias de sus intestinos producen las enzimas que degradan la celulosa de la que se alimentan (Warnecke et al., 2007); las bacterias de las glándulas salivales de la hormiga león (*Myrmeleon bore*), producen una toxina que el insecto inyecta a sus presas para paralizarlas y capturarlas (Yoshida et al., 2001); los escarabajos del género *Paederus* poseen simbiontes que producen pederina, una toxina que utilizan como defensa contra depredadores (Piel, 2002).

Cuando los simbiontes son eliminados de los insectos, éstos presentan un fenotipo anormal. La expresión de genes involucrados en el sistema inmune como *DUOX, domeless* y *caudal,* es significativamente menor en Moscas tsetse (*Glossina morsitans morsitans*) que no tenían sus simbiontes nativos (*Wigglesworthia, Sodalis* y *Wolbachia*). Además, muestran mayor susceptibilidad a microorganismos extraños que las moscas con microbiota normal. (Weiss et al., 2012). En chinches apestosas de la especie *Elasmostethus humeralis,* la tasa de emergencia es significativamente menor, el tiempo de desarrollo para llegar a la adultez es más largo y el patrón de coloración es anormal en individuos provenientes de huevecillos esterilizados en los que se eliminaron los simbiontes (Kikuchi et al., 2009) (**Fig. 1**). Igualmente, en insectos de la especie *Riptortus pedestris*, plaga de cultivos como soya o frijol, la eliminación de la *Burkholderia* simbionte tiene un efecto perjudicial en sus hospederos, ya que hay una diferencia significativa en la longitud del cuerpo de los insectos, el ancho del tórax y el tiempo que les toma llegar a la edad adulta (Kikuchi & Fukatsu, 2013).

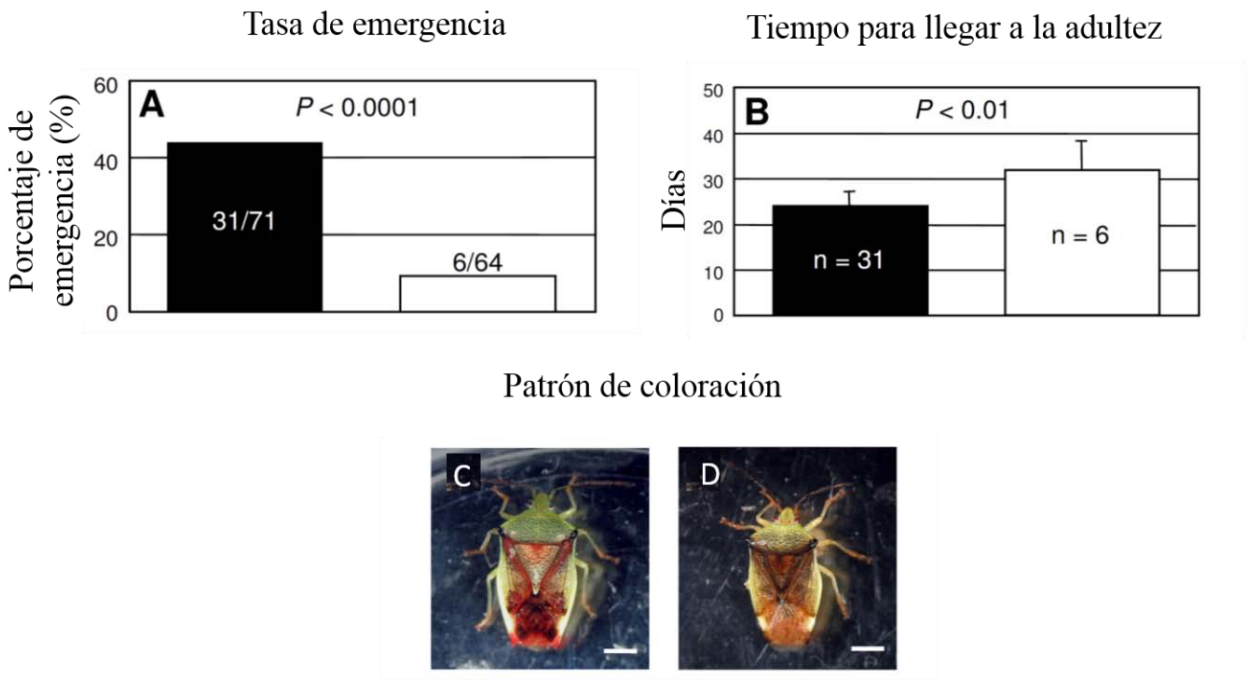

Figura 1. Efectos de la eliminación de los simbiontes en el fenotipo de *Elasmostethus humeralis*. A) Tasa de emergencia de los insectos (%). Insectos que emergieron/ total de insectos. B) Tiempo de desarrollo a la edad adulta (días). C) Hembra adulta del tratamiento control mostrando un patrón de coloración normal. D) Hembra adulta libre de simbiontes del tratamiento de huevecillos esterilizados mostrando patrón de coloración anormal. Barra, 2.5 mm. (Modificado de Kikuchi et al., 2009). Barras negras: insectos control; Barras blancas: insectos libres de simbiontes.

Bacterias endosimbiontes

Las relaciones simbióticas entre insectos y bacterias llegan a ser tan estrechas que la simbiosis es necesaria para la sobrevivencia de ambas especies (Douglas, 1998). Cuando las bacterias viven dentro de células del insecto se conocen como endosimbiontes (Gil et al., 2004). Los endosimbiontes se albergan en células llamadas bacteriocitos que se agregan en estructuras en el abdomen conocidas como bacteriomas o pueden encontrarse en otros tejidos como cuerpo graso o hemolinfa (**Fig. 2**).

Durante el proceso de endosimbiosis las bacterias se ven sometidas a una nueva dinámica poblacional. Generalmente, la transmisión de los endosimbiontes ocurre de manera vertical por línea materna (Moran, 2007), lo que ocasiona que sólo una parte de la población sea transmitida a la siguiente generación propiciando cuellos de botella que reducen la posibilidad de intercambio genético y resultan en la acumulación de mutaciones deletéreas (Moya et al., 2008). En los endosimbiontes ocurren cambios genéticos resultado de su nuevo estilo de vida, por ejemplo, pierden genes que ya no son esenciales para su supervivencia como los de síntesis de pared celular, ya que comúnmente se encuentran protegidos dentro de células del insecto; también pierden genes para la síntesis de nutrientes que ahora les provee el hospedero. Debido a esto sufren una reducción en el tamaño de su genoma quedando entre 138 a 1500 kb. El genoma del endosimbionte de las cochinillas harinosas es el más pequeño que se conoce, la β-proteobacteria *Candidatus* Tremblaya princeps que tiene un tamaño de 138, 927 pb (McCutcheon & von Dohlen, 2011). El uso de codones es distinto, incluyendo el codón de terminación, y tienden a tener un contenido de AT mayor al 50%, excepto en el caso de *Candidatus* Hodgkinia cicadicola, simbionte de la chicharra *Diceroprocta semicincta*, que posee un genoma de 144 kb con un contenido de GC de 58% (McCutcheon et al., 2009b). El establecimiento de una asociación permanente depende de la

evolución de mecanismos adecuados para las interacciones metabólicas y regulatorias entre simbiontes y hospedero. Por ejemplo, las bacterias necesitan adaptar su replicación para que su tasa de crecimiento esté coordinada con el desarrollo de sus hospederos como en el caso de *Buchnera aphidicola* en la cual el tiempo de replicación de es de aproximadamente 2 días, un periodo mucho más largo que el de las bacterias de vida libre que llega a ser tan corto como 30 minutos. Otras bacterias endosimbiontes como *Blochmania floridanus* en las hormigas, y *Wigglesworthia glossinidia* en las moscas Tsetse, carecen del gen *dnaA*, el cual es esencial en bacterias ya que codifica para la proteína de iniciación de la replicación. Es posible que exista un control más directo de la replicación de los simbiontes por el hospedero, involucrando la pérdida de las proteínas esenciales para la iniciación de la replicación (Gil et al., 2004).

El repertorio genético que presentan los genomas de endosimbiontes está en función de las características particulares de cada asociación como el aporte que hace la bacteria al insecto para compensar sus deficiencias nutricionales (Baumann, 2005).

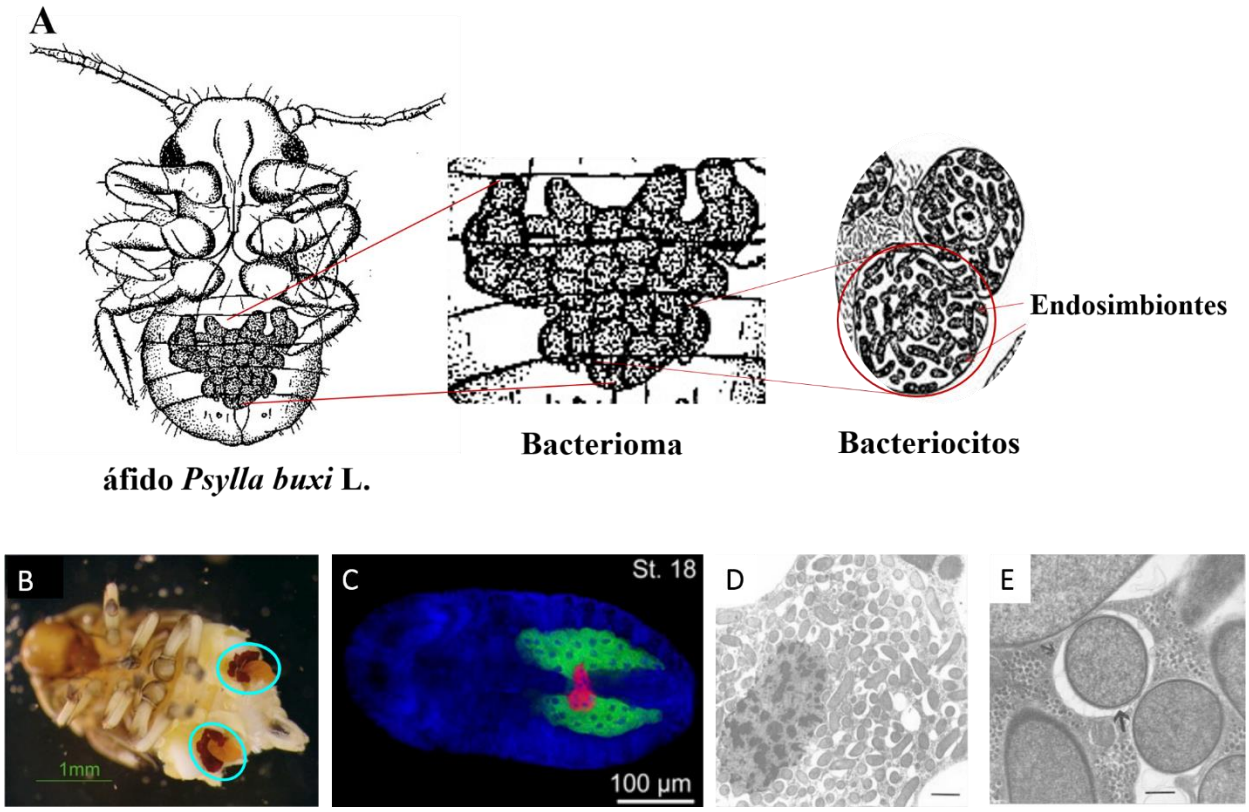

Figura 2. Estructuras que albergan los endosimbiontes. A) Esquema del bacterioma del áfido *Psylla buxi* L. que muestra los endosimbiontes dentro de bacteriocitos. (Modificado de Buchner, 1965); B) Par de bacteriomas en el abdomen de *Clastoptera arizonana* (círculos azules) (Moran, 2006); C) Hibridación fluorescente *in situ* de los simbiontes *Buchnera* y *Serratia* en el bacterioma de un embrión del estadío 18 del áfido *Acyrtosyphon pisum*. Verde: *Buchnera*; Rojo: *Serratia*; Azul: núcleos del insecto (Modificado de Koga et al., 2012); D) Micrografías del endosimbionte *Blattabacterium cuenotti* dentro de bacteriocitos del cuerpo graso de la cucaracha *Periplaneta americana* (barra= 3.0 µm) (Modificado de Bauman et al. 2013); E) Ultraestructura del endosimbionte de la cucaracha *Cryptocercus punctulatus* que muestra una pared celular típica Gram-negativa (flecha grande) y la membrana vesicular que contiene a la bacteria (flecha pequeña) (barra= 0.3 µm) (Modificado de Bauman et al. 2013).

Endosimbiontes del género *Wolbachia*

Las bacterias del género *Wolbachia* son endosimbiontes que colonizan nemátodos de la familia Onchocercidae (Comandatore et al., 2013; Koutsovoulos et al., 2014) y diferentes grupos de artrópodos (Hilgenboecker et al., 2008). No tienen una fase de vida libre y están filogenéticamente relacionados con bacterias intracelulares del Orden Rickettsiales que incluyen patógenos con genomas reducidos (Blanc et al., 2007). Se estima que *Wolbachia* infecta entre el 40 (Zug & Hammerstein, 2012) al 60% de las especies de artrópodos (Hilgenboecker et al., 2008).

Sus efectos en sus hospederos varían desde mutualismo hasta parasitismo (Hilgenboecker et al., 2008; Sommer & Streit, 2011). En nemátodos, *Wolbachia* causa beneficios mediante la provisión de vitaminas y energía y ayudando a la evasión del sistema inmune del hospedero del nemátodo (Darby et al., 2012) y se consideran esenciales para su supervivencia (Landmann et al., 2014). En ácaros como *Cimex lectularius*, *Wolbachia* provee biotina (Nikoh et al., 2014); en *Drosophila* puede conferir protección contra virus (Chrostek et al., 2013; Teixeira et al., 2008) e incrementar la fecundidad de las hembras (Brownlie et al., 2009); en avispas de la especie *Asobara tabida* la remoción de *Wolbachia* inhibe la maduración de los oocitos (Dedeine et al., 2001). En contraste, en otros insectos manipula la reproducción mediante partenogénesis (Huigens et al., 2000), incompatibilidad citoplasmática (Rousset et al., 1992), "male-killing" (Duplouy et al., 2013; Zeh & Zeh, 2006) o feminización (Rousset et al., 1992).

La transmisión de *Wolbachia* en los artrópodos ocurre verticalmente, sin embargo, las infecciones de *Wolbachia* también pueden ocurrir de manera horizontal entre individuos de la misma o de diferentes especies. Se cree que este tipo de transmisión es poco común, pero relaciones como las de avispas parasitarias con sus hospederos pueden propiciarla (Vavre et al., 1999).

ANTECEDENTES

Biología del género *Dactylopius*

Dactylopius es un género de insectos originario de América perteneciente a la familia Dactylopiidae dentro de la superfamilia Coccoidea del orden Hemiptera. Comúnmente se les conoce como cochinilla del nopal o grana cochinilla (**Fig. 3**). Son parásitos que se alimentan únicamente de la savia de plantas del género *Opuntia* (Cactaceae), también conocidas como nopales.

Los insectos del género *Dactylopius,* principalmente *Dactylopius coccus* (Costa)*,* son la fuente del ácido carmínico (Dapson, 2005), colorante que ha sido aprovechado desde la época prehispánica (Flores-Coronado, 2011), y que sigue siendo utilizado en la industria alimentaria, textil y cosmética (Ramírez-Cruz et al., 2008), donde representa una alternativa a los colorantes sintéticos asociados con carcinogénesis. Además, por sus hábitos parasitarios, las especies silvestres de *Dactylopius* como *D. opuntiae* y *D. tomentosus* son usadas como control biológico contra opuntias invasoras en Australia y algunos países de África donde desplazan plantas nativas (Mathenge et al., 2009b)

Hasta la fecha se han descrito once especies de *Dactylopius*: *Dactylopius austrinus, D. bassi, D. ceylonicus, D. coccus, , D. confertus, D. confusus, D. gracilipilus, D. opuntiae, D. salmianus, D. tomentosus,* y *D. zimmermani*, que actualmente se encuentran distribuidas a nivel mundial ya que han sido introducidos en diferentes regiones geográficas (Chávez-Moreno et al., 2009; Van Dam & May, 2012). Cinco de estas especies (*D. ceylonicus, D. coccus, D. confusus, D. opuntiae y D. tomentosus)* están presentes en México (Portillo & Vigueras, 2006; Ramírez-Cruz et

al., 2008). Todavía existe controversia respecto a la taxonomía de las especies ya que por su parecido morfológico no se puede discernir con seguridad entre algunas de ellas, por ejemplo, *D. ceylonicus* y *D. opuntiae* llegan a confundirse como una sola especie. No existen estudios moleculares sobre la filogenia de estos insectos que confirmen o refuten la clasificación actual.

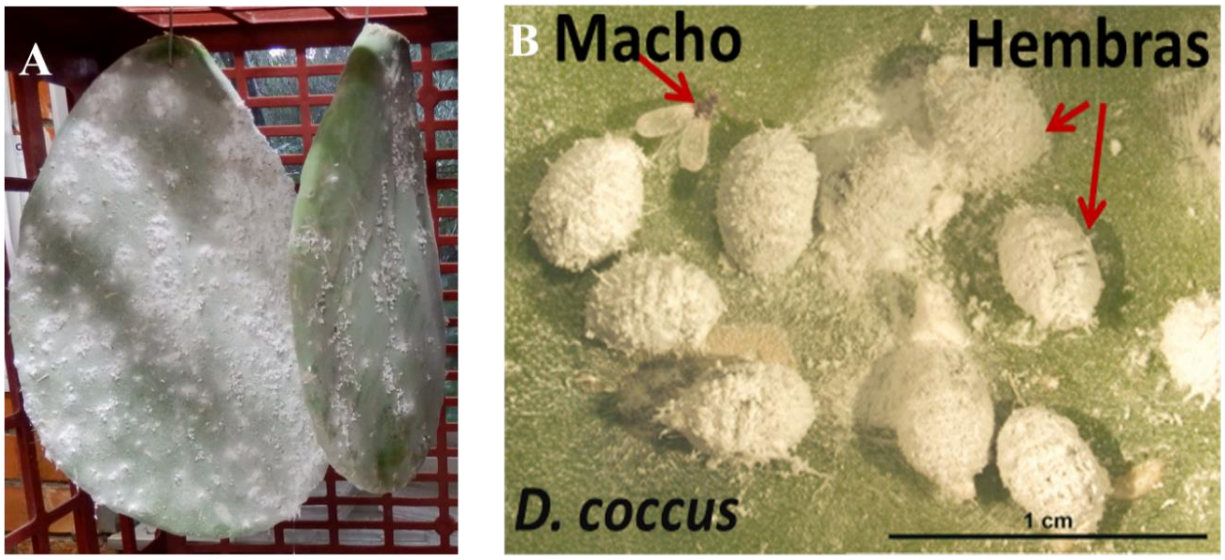

Figura 3. Insectos de la especie *Dactylopius coccus*. A) Hembras adultas alimentándose sobre un cladodio de *Opuntia ficus-indica*. B) Fotografía de insectos adultos en la que se observan los machos alados y las hembras sésiles.

Ciclo de vida de la grana cochinilla

Su ciclo de vida es de aproximadamente 110 días y se divide en tres etapas de desarrollo: huevecillo, ninfa y adulto. Estos insectos son hemimetábolos, es decir, las hembras no llevan a cabo metamorfosis completa (Flores-Coronado, 2011), por lo que ninfas y adultas tienen un aspecto muy parecido distinguiéndose solamente por el tamaño. En los primeros días después de la eclosión, las ninfas caminan sobre la superficie del cladodio hasta encontrar un lugar donde insertar el estilete y una vez que esto ocurre nunca más vuelven a sacarlo, transcurriendo toda su vida en el mismo lugar sobre la planta. Las hembras adultas son sésiles y pueden medir hasta 6 mm mientras que los machos desarrollan alas y llegan a medir 3 mm. Pasan por dos estadíos ninfales: ninfa I los primeros 36 días; ninfa II hasta el día 56 y 46 hembras y machos, respectivamente; los machos atraviesan un estadio de pupa del día 47 al 57. A los 57 días las hembras alcanzan la edad adulta y los machos a los 58 días (**Fig. 4**). En esta etapa ocurre la cópula, las hembras son fecundadas y los machos mueren entre tres y cinco días después, viviendo sólo el tiempo suficiente para fecundarlas. A los 90 días las hembras ovipositan de 150-200 huevecillos que tienen un periodo de incubación de 24h, excepto *D. tomentosus* que se prolonga hasta 17 días (Mathenge et al., 2009a). Pocas semanas después de la ovoposición las hembras adultas mueren.

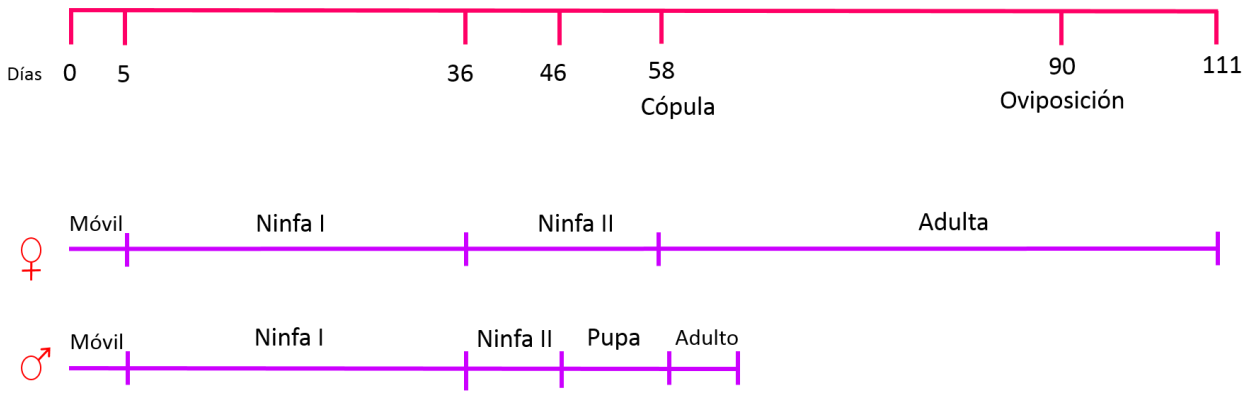

Figura 4. Ciclo de vida de la grana cochinilla. Después de eclosionar, las ninfas pasan por una etapa móvil que dura aproximadamente 5 días. El estadio de ninfa I dura hasta los 36 días; el estadio de ninfa II dura hasta los 57 días en hembras y 46 días los machos. A los 47 días los machos entran al estadio de pupa y alcanzan el estado adulto a los 58 días cuando ocurre la cópula. El ciclo termina a los 63 días y 111 días en machos y hembras, respectivamente.

PLANTEAMIENTO DE PROBLEMA

Las opuntias son plantas que crecen en climas áridos de zonas tropicales y subtropicales y comprenden entre 200-300 especies. Se caracterizan porque los tallos han sustituido a las hojas en su función fotosintética. Los constituyentes principales de los tallos por cada 100g de peso fresco son: agua (88-95%), carbohidratos (3-7%), fibra (1-2%), lípidos (0.2 %) y proteínas (0.5-1%)(Stintzing & Carle, 2005). El contenido nutritivo depende del sitio de cultivo, estación del año y edad de la planta por lo tanto pueden variar entre cultivares, sin embargo, por su alto porcentaje de agua (88- 95%) se consideran un alimento bajo en calorías (Ginestra et al., 2009).

En 2007, Pankewitz y colaboradores reportaron la presencia de *Wolbachia* sp. en huevecillos de *Dactylopius* sp., pero no se realizaron análisis más exhaustivos sobre los simbiontes de estos insectos.

Dado que las cochinillas del nopal se alimentan únicamente de la savia de estas cactáceas y que los insectos que se alimentan de este tipo de dietas frecuentemente mantienen relaciones simbióticas con bacterias que les proveen nutrientes para compensar las deficiencias de su dieta, podemos esperar que los insectos del género *Dactylopius* establezcan simbiosis con bacterias.

HIPÓTESIS

Los insectos del género *Dactylopius* tienen una dieta basada en savia la cual es pobre en aminoácidos y vitaminas, por lo que sus simbiontes bacterianos pueden estar complementando sus requerimientos nutricionales.

OBJETIVO GENERAL

Identificar los simbiontes bacterianos de *Dactylopius* y analizar los genomas de algunos para inferir cuál es el aporte de éstos al hospedero.

Objetivos particulares

- Identificar morfológica y molecularmente las especies de las muestras de *Dactylopius* colectadas.
- Identificar las bacterias que se encuentran asociadas al género *Dactylopius*.
- Describir el papel juegan las bacterias simbiontes en la asociación con el insecto mediante el análisis de su genoma.

RESULTADOS

A continuación se presentan 3 artículos en los que se relatan los resultados más importantes producto de este trabajo de investigación. Dos de ellos**, "Molecular phylogeny of the genus *Dactylopius*, and identification of the symbiotic bacteria"** y **"Species in *Wolbachia*? Proposal for the designation of '*Candidatus* Wolbachia bourtzisii', '*Candidatus* Wolbachia onchocercicola', '*Candidatus* Wolbachia blaxteri', '*Candidatus* Wolbachia brugii', '*Candidatus* Wolbachia taylori', '*Candidatus* Wolbachia collembolicola' and '*Candidatus* Wolbachia multihospitium' for the different species within *Wolbachia* supergroups** ya han sido publicados y el tercero, **"Two *Wolbachia* strains, *w*DacB and *w*DacA, from the metagenome of the cochineal insect *Dactylopius coccus* (Hemiptera: Dactylopiidae)"**, se encuentra en preparación para ser enviado para revisión próximamente.

Molecular Phylogeny of the Genus *Dactylopius* (Hemiptera: Dactylopiidae) and Identification of the Symbiotic Bacteria

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ABSTRACT Phylogenetic analyses, from polymerase chain reaction (PCR)-amplified 12S rRNA and 18S rRNA gene sequences from cochineal insects of the genus *Dactylopius* present in Mexico, showed that *D. ceylonicus*, *D. confusus*, and *D. opuntiae* are closely related. *D. coccus* constitutes a separate clade, and *D. tomentosus* is the most distantly related. Bacterial 16S rRNA sequences from all the *Dactylopius* species sampled showed a common β-proteobacteria, related to *Azoarcus*, also found in eggs and in bacteriocytes in *D. coccus*. We propose the name "*Candidatus* Dactylopiibacterium carminicum" for this endosymbiont. Other bacterial sequences recovered from the samples were close to those from soil or plant associated bacteria, like *Massilia*, *Herbaspirillum*, *Acinetobacter*, *Mesorhizobium*, and *Sphingomonas*, suggesting a possible horizontal transmission from Cactaceae plant sap to *Dactylopius* spp. during feeding. This is the first molecular analysis of *Dactylopius* species and of their associated bacteria.

RESUMEN Análisis filogenéticos de secuencias amplificadas mediante PCR de los genes 12S rARN y 18S rARN de insectos cochinillas del género *Dactylopius* presentes en México, mostraron que *D. ceylonicus*, *D. confusus* y *D. opuntiae* están cercanamente relacionados. *Dactylopius coccus* constituye un clado separado y *D. tomentosus* es el más alejado. Las secuencias del 16S rARN de bacterias de las especies de *Dactylopius* revelaron una β-Proteobacteria común, relacionada a *Azoarcus*, también encontrada en huevecillos y en bacteriocitos de *D. coccus*. Proponemos el nombre de "*Candidatus* Dactylopiibacterium carminicum" para este endosimbionte. Otras secuencias bacterianas recuperadas de las muestras fueron cercanas a bacterias del suelo o asociadas a plantas, como *Massilia*, *Herbaspirillum*, *Acinetobacter*, *Mesorhizobium* y *Sphingomonas*, sugiriendo que estas bacterias fueron transferidas de manera horizontal de la savia de las cactáceas a *Dactylopius* spp. durante la alimentación. Este es el primer análisis molecular de especies *Dactylopius* y de sus bacterias asociadas.

KEY WORDS Coccoidea, scale insects, systematics, endosymbiont, bacteriocytes

Dactylopius (Costa) is a genus of insects commonly known as cochineals that belongs to the family Dactylopiidae (Signoret) from the super family Coccoidea (scale insects) within the order Hemiptera. *Dactylopius* insects feed on Cactaceae plants from the genera *Opuntia* and *Nopalea* (Pérez-Guerra and Kosztarab 1992). Both the insects and their host plants are native to the Americas (Pérez-Guerra and Kosztarab 1992, Chávez-Moreno et al. 2009). Dactylopiidae has only one genus that includes nine described species (De Lotto 1974, Pérez-Guerra and Kosztarab 1992). Five of these species have been reported to be present in Mexico: *D. ceylonicus*, *D. confusus*, *D. opuntiae*, *D. coccus*, and *D. tomentosus* (Portillo and Vigueras 2006, Chávez-Moreno et al. 2009). *Dactylopius* spp. produce carminic acid, which is used by the insects for protection against predators (Eisner et al. 1994). It is used as a red dye for the production of cosmetics, drugs, food, and textiles. *D. coccus* has been preferentially used for carminic acid extraction because of its pigment quality and higher acid content (Hernández-Hernández et al. 2005). There are reports of its use in America since the 10th century (Portillo 2005, Chávez-Moreno et al. 2009). Currently, *D. coccus* is considered the only commercially important species in this genus, and it has undergone a domestication process. This species depends on human care for dispersion and reproduction (Pérez-Guerra and Kosztarab 1992). *Dactylopius* spp. have also been used as a biological control agent against

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invasive cactus in Africa and Australia (Moran and Zimmermann 1984).

Many insects harbor symbiotic bacteria in their guts or as endosymbionts inside specialized insect cells called bacteriocytes (Baumann 2005). Bacterial endosymbionts in the Hemiptera provide nutrients to insects with a limited diet such as phloem sap and blood that are deficient in essential amino acids and vitamins (Baumann 2005, Moran 2006). Some endosymbionts also synthesize bioactive compounds that can be used by insect hosts as defense against predators, parasites, and pathogenic microorganisms (Moran 2006). As endosymbionts are vertically transmitted, their DNA sequences can be used to trace insect phylogenies (Baumann 2005). Within the Coccoidea, endosymbionts have been found in the families Pseudococcidae (Thao et al. 2002), Diaspididae, and Margarodidae (Gruwell et al. 2007). The diversity of endosymbionts in *Dactylopius* spp. has not been reported, except for bacteria from the genus *Wolbachia* present in *Dactylopius* sp. eggs (Pankewitz et al. 2007).

The current identification and taxonomy of *Dactylopius* spp. has been based on morphological characters (De Lotto 1974, Pérez-Guerra and Kosztarab 1992), and Rodríguez et al. (2001) published a phylogeny of *Dactylopius* spp. on this basis. Until now, there have been no molecular phylogenies of the genus. There is only one phylogeny based on 18S rRNA sequences of the Coccoidea, which places Dactylopiidae close to clade E1 from the Eriococcidae (Cook et al. 2002). The aims of this work were to sequence and analyze mitochondrial and nuclear ribosomal genes from *Dactylopius* spp. to assess the phylogenetic relationships between the five species present in Mexico and to determine the symbiont bacteria species present in these insects.

Materials and Methods

Insect Sampling. Specimens from five different *Dactylopius* species were collected from different regions in Mexico. Adult females were slide-mounted to allow identification according to descriptions given by De Lotto (1974) and Pérez-Guerra and Kosztarab (1992). Vouchers were deposited in the Colección Nacional de Insectos of the Instituto de Biología or in Centro de Ciencias Genómicas of the Universidad Nacional Autónoma de México. Specimens were collected from the following states (voucher numbers are indicated in parentheses): *D. coccus* from Oaxaca (DTY-ChM-101) and Morelos (Campo Carmín) (CCG-Sham-1), *D. confusus* from Tlaxcala (DTY-ChM-132), *D. ceylonicus* from Mexico state (DTY-ChM-110), *D. opuntiae* from Michoacán (DTY-ChM-106) and Querétaro (CCG-Cacau-2), and additionally we obtained *D. opuntiae* from Brazil, Pernambuco state (CCG-Cacau-8); all of these were parasitizing *Opuntia ficus-indica* L. Miller plants. *Dactylopius tomentosus* (DTY-ChM-190) was collected from Hidalgo on *Cylindropuntia tunicata* (Lehmann) Knuth, *Parasaissetia* sp. (Hemiptera: Coccidae) insects (CCG-AL-8) were collected in Morelos state on *Jacaranda mimosifolia* (D. Don). Gene sequences derived from this Coccidae were considered an outgroup in the phylogenetic analyses.

DNA Extraction, Amplification, and Sequencing. Female specimens from each insect species, freshly collected or frozen (−20°C), were superficially cleaned, removing the white wax, washed, and vortexed several times with ethanol and rinsed with sterile distilled water. DNA was extracted from whole insects with DNeasy Blood and Tissue Kit (Qiagen Hilden, Germany). A sample represented one specimen in the case of *D. coccus* and two to four specimens from the other species. Two DNA samples were analyzed from each species. DNA was used as a template in PCR reactions using primers F-12S-2: 5′-AAGAGTGACGGGCRATTTGTACATA-3′ and R-12S-2: 5′-GTGCCAGCAGTWGCGGTTA-3′ for insect mitochondrial 12S rRNA gene (Thao et al. 2004), primers 2880: 5′-CTGGTTGATCCTGCCAGTAG-3′ (Tautz et al. 1988) and B-: 5′-CCGCGGCTGCTGGCACCAGA-3′ (von Dohlen and Moran 1995) for insect nuclear 18S rRNA gene, and primers fD1: 5′-AGAGTTTGATCCTGGCTCAG-3′ and rD1: 5′-AAGGAGGTGATCCAGCC-3′ for bacterial 16S rRNA gene (Weisburg et al. 1991). Eggs and bacteriocytes were dissected from individual *D. coccus* females and washed several times with phosphate-buffered saline (PBS: 120 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, [pH 7.4]), and DNA was extracted and used in PCR reactions as described above. Primers that specifically amplify a fragment from the 16S rRNA gene of END1 and closely related β-proteobacteria were designed (Beta428 F: 5′-GTGAATATCCGAAGCCGATGAC-3′, Beta1205R: 5′-GGCTTGGCAACCCTCTGTACCG-3′). Primers to identify clone O1 and related α-Proteobacteria were also designed (Alpha141 F: 5′-ACGGAAGAAAGTAGATATACGC-3′ and Alpha944R: 5′-ACCTGTTATGCTCCAACTAAAT-3′). These were used in addition to fD1 and rD1 primers with DNA of *Dactylopius* spp.

PCR protocols were performed as described by Weisburg et al. (1991) and Thao et al. (2004), except for the 18S rRNA gene that was amplified using the procedures described by Cook et al. (2002). The following protocol was used with Beta428 F–Beta1205R and Alpha141 F–Alpha944R primers: an initial denaturation at 94°C for 3 min, followed by 33 cycles of amplification (1 min at 94°C, 1 min at 57 or 52°C, respectively, and 1 min at 72°C), and a final extension step of 5 min at 72°C. The amplified products were 1,500 (fD1 and rD1), 460 (F-12S-2 and R-12S-2), 620 (2880 and B-), 800 (Beta428 F and Beta1205R), and 825 bp (Alpha141 F and Alpha944R). PCR products were cloned and individual plasmid clones were sequenced in Macrogen (Seoul, Korea).

Bacterial Culture Conditions. *Dactylopius coccus* were superficially cleaned, and macerate extracts were plated in (LB) (Sambrook and Russell 2001) and (PY) medium (Noel et al. 1984) and grown for 21 d at 28°C.

Phylogenetic Analyses. Nucleotide sequences were compared using the GenBank database Blastn, and

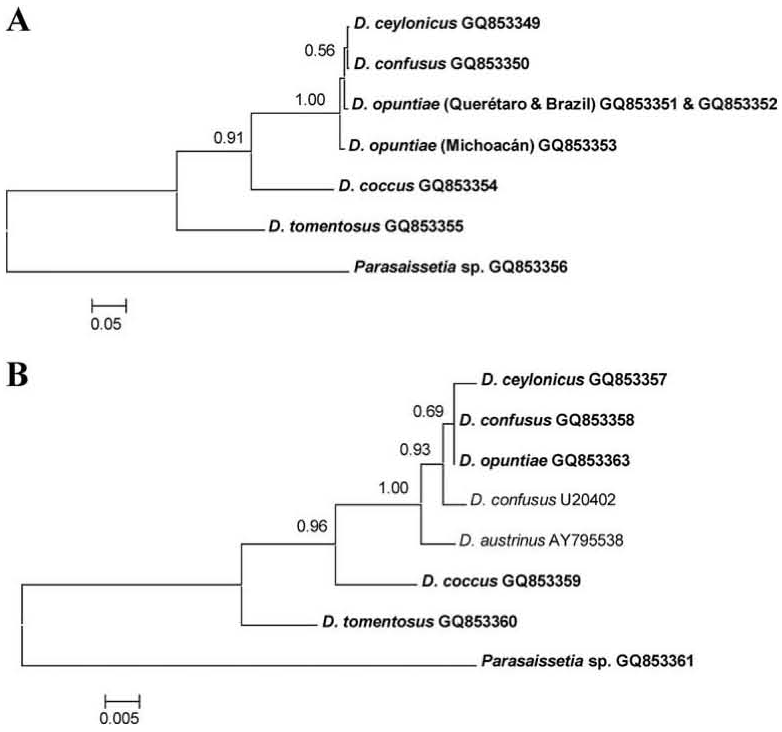

Fig. 1. Phylogenetic trees of 12S rRNA gene sequences (426 bp) (A) and 18S rRNA gene sequences (584 bp) (B) obtained from different *Dactylopius* species. Sequences from this work are in bold. Accession numbers are shown after scientific names. The tree was inferred with the Bayesian method using MrBayes under model GTR+I+G. Numerical values at each node indicate posterior probabilities. *Parasaissetia* sp. gene sequences were used as outgroups.

sequences from closely related organisms were retrieved. Sequence alignments were performed with CLUSTAL W (Thompson et al. 1994) and manually edited. Phylogenies were constructed with the Bayesian method using MrBayes (Huelsenbeck and Ronquist 2001). The best model of sequence evolution for each gene was selected using the MrAIC Perl script written by J.A.A. Nylander (http://www.abc.SE/≈nylander/). In all cases, the selected model was GTR+I+G. *Candidatus* Sulcia muelleri 16S rRNA gene sequence was used as an outgroup; this is an endosymbiotic flavobacterium of many species of the suborder Auchenorrhyncha of Hemiptera (Moran et al. 2005).

Results

Phylogenetic Analyses. Six clones from each *Dactylopius* species were analyzed (three clones per sample for each gene analyzed). Sequences obtained from a single insect species were >99% identical, and only one clone from each species was used for phylogenetic analyses. Phylogenetic trees obtained from the mitochondrial 12S rRNA and nuclear 18S rRNA genes are shown in Fig. 1A and B. A tree generated with concatenated sequences from both genes was similar (data not shown). Trees were congruent and showed that *D. ceylonicus*, *D. confusus*, and *D. opuntiae* clustered together with an identity of 99%. 12S rRNA gene sequences from *D. opuntiae* from three different geographic regions were very similar (>98.9% of identity), and 18S rRNA gene sequences from *D. confusus* and from the three *D. opuntiae* were 100% identical. Two 18S rRNA gene sequences were retrieved from NCBI GenBank corresponding to *D. confusus* [U20402, collected from Arizona (von Dohlen and Moran 1995)] and *D. austrinus* [AY795538, collected from Australia (Cook and Gullan 2004)]. The *D. confusus* sequence is similar to those reported here for *D. ceylonicus*, *D. confusus*, and *D. opuntiae* (99% of identity), and *D. austrinus* sequence was close to this cluster. *D. coccus* was separated, and *D. tomentosus* was the most distantly related.

Identification of Symbiotic Bacteria. The analyses of 16S rRNA gene sequences indicated that different bacteria were found inside *Dactylopius*. The abundance of clones found in each species and the percentage of identity to the closest NCBI match are shown in Table 1.

Dactylopius species had different associated bacteria; however, the only universally associated one was a β-proteobacteria (named here as END1) and was highly conserved with almost identical 16S rRNA gene sequences. END1 was first identified with 16S rRNA universal primers (fD1 and rD1) in all the individuals collected from *D. opuntiae*, including the ones collected from Brazil, and in *D. coccus* and *D. tomentosus* (Table 1). Subsequently, with primers specific to END1 (Beta428 F and Beta1205R), it was also found in *D. ceylonicus* and *D. confusus*, as well as in eggs and

Table 1. Assignment and abundance (percentage of clones) of bacteria in *Dactylopius* species using 16S rRNA gene sequences obtained with universal primers rD1 and fD1

Dactylopius species	β-Proteobacteria			α-Proteobacteria			γ-Proteobacteria	Number of analyzed clones
	Soil bacteria (END1)	*Massilia* sp.(N6)	*Herbaspirillum* sp. (E7)	*Porcellio scaber* symbiont (O1)	*Sphingomonas insulae* (E1)	*Mesorhizobium* sp. (E4)	*Acinetobacter* sp. (N8)	
D. ceylonicus	0[a]	29	43	0	14	14	0	7
D. confusus	0[a]	88	0	0	0	0	12	8
D. opuntiae (Michoacán)	100	0	0	0	0	0	0	2
D. opuntiae (Querétaro)	63	0	0	37	0	0	0	27
D. opuntiae (Brazil)	43	0	0	57	0	0	0	7
D. coccus	100	0	0	0	0	0	0	10
D. tomentosus	100	0	0	0	0	0	0	6

The closest NCBI match is shown. Name of each clone is shown in parentheses.

[a] END1 symbiont was not found when universal 16S rRNA gene primers (rD1 and fD1) were used, but it was amplified by PCR when specific primers for β-proteobacteria were used (Beta428F and Beta1205R).

bacteriocytes of *D. coccus*. The sequence identity of END1 from the different *Dactylopius* species was >99.5%. The sequence had 95% identity to a soil isolate (AB024934) that was erroneously assigned to *Sphingomonas* sp. A1 (α-proteobacteria) and corresponds to a sequence of β-proteobacteria closely related to *Azoarcus* sp. (Fig. 2). END1 sequence also presents 95% identity to *Uliginosibacterium gangwonense*, an aerobic bacteria isolated from wetland peat samples (Weon et al. 2008). No cultured isolates were obtained from *D.*

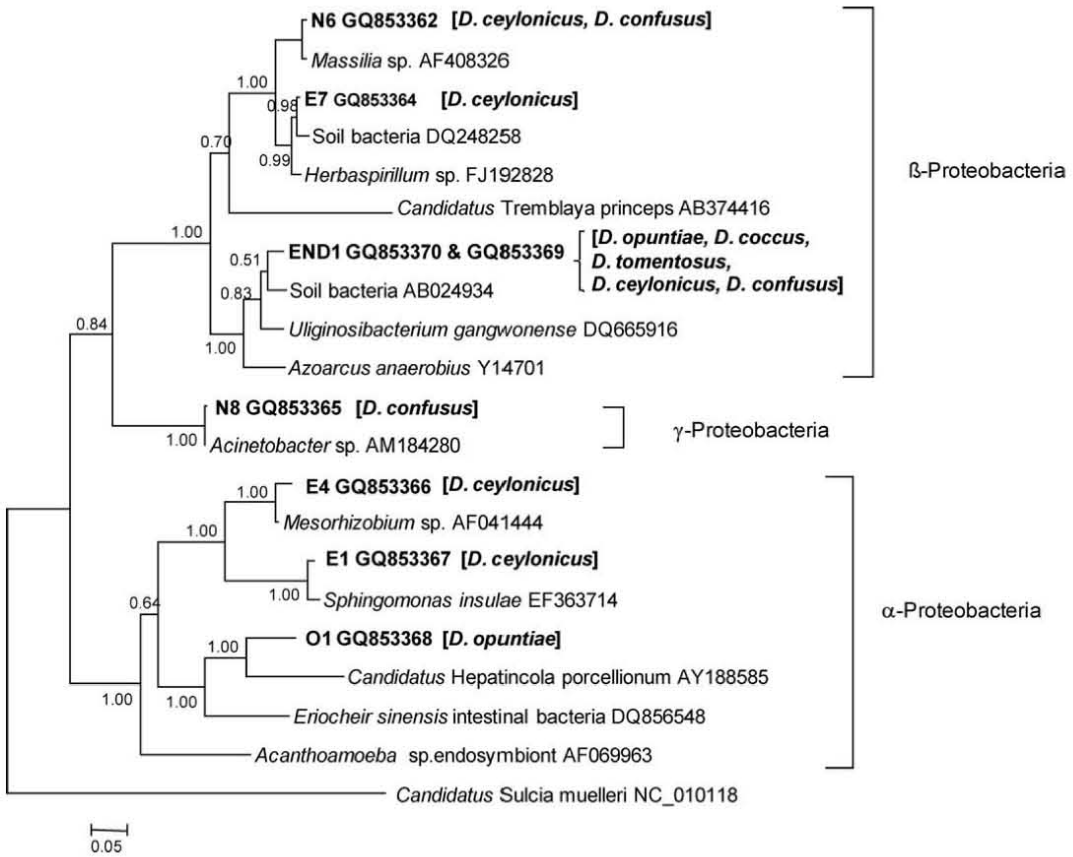

Fig. 2. Phylogenetic tree of 16S rRNA gene sequences of bacteria (1284 bp) obtained from different *Dactylopius* species. Sequences from this work are in bold. Other sequences from closely related organisms were included. Accession numbers are shown after scientific names. Host *Dactylopius* species are shown in brackets. The tree was inferred with the Bayesian method using MrBayes under model GTR+I+G. Numerical values at each node indicate posterior probabilities. *Candidatus* Sulcia muelleri 16S rRNA gene sequence was used as outgroup.

coccus female macerates plated in LB and PY medium (data not shown).

Additionally, 39% of the 16S ribosomal RNA clones obtained from the DNA samples of all *D. opuntiae* specimens presented 88% identity to *Candidatus* Hepatincola porcellionum, the extracellular symbiont of the hepatopancreas of *Porcellio scaber* (common woodlouse, Crustacea: Isopoda) (Wang et al. 2004) (clone O1 in Table 1 and Fig. 2), which belongs to the order Rickettsiales from the α-proteobacteria. Specific primers for O1 did not amplify DNA from other *Dactylopius* species.

Other 16S rRNA gene clones were close to free-living bacteria, such as *Massilia* sp., *Herbaspirillum* sp., *Acinetobacter* sp., *Mesorhizobium* sp., and *Sphingomonas* sp. (Table 1; Fig. 2), with 98, 96, 99, 97, and 98% identity, respectively. These were obtained from *D. ceylonicus* and *D. confusus*.

Discussion

Our phylogenetic results on the relationships of species in the cochineal genus *Dactylopius* differ from those reported by Rodríguez et al. (2001) based on morphological characters. They found that *D. austrinus*, *D. ceylonicus*, and *D. coccus* are more closely related between them and less related to *D. confusus* and *D. opuntiae*. The need to establish molecular phylogenies for *Dactylopius* has been recognized in several papers because conflicting results have been derived from morphological data (Portillo and Vigueras 2006).

Dactylopius coccus was domesticated and selected for producing high amounts of carminic acid. It has a large body size (females are 3–6 mm long) and presents a cover of white powdery wax instead of a white cottony wax with long filaments (Pérez-Guerra and Kosztarab 1992). The cottony wax cover protects the insects against desiccation and rain (Chávez-Moreno et al. 2009). Pérez-Guerra and Kosztarab (1992) considered the characteristic cover important for proposing *D. coccus* as the most primitive of the *Dactylopius* species; otherwise, this character could be a consequence of domestication.

In accordance with our results, *D. tomentosus* has been reported as the most distant species of the genus, because it has unique biological and morphological characteristics that differ considerably from other *Dactylopius* species (Mathenge et al. 2009). *Dactylopius tomentosus* host range is restricted to the subgenus *Cylindropuntia*, its egg incubation period is longer (17 d instead of minutes or hours), eggs are held on a mesh of waxy threads and remain attached to the female during the incubation period (in other *Dactylopius* species, eggs are not enclosed in a mesh and continue to hatch as more are laid), the size of female adults is smaller than in most of the other species (Mathenge et al. 2009), and its anal ring is obsolete (Pérez-Guerra and Kosztarab 1992, Rodríguez et al. 2001).

We found a characteristic set of bacteria from each *Dactylopius* species. Of special interest is END1, a β-proteobacteria found in all the *Dactylopius* species and in eggs and bacteriocytes of *D. coccus*. These findings suggest that END1 is a primary endosymbiont that could have been acquired before the radiation of this genus. Its location should be subsequently confirmed by in situ hybridization. Within the Coccoidea in the Pseudococcidae family, another β-proteobacteria primary endosymbiont, *Candidatus* Tremblaya princeps, has been reported (Thao et al. 2002), with 16S rRNA gene sequence 79% identical to that from END1 (Fig. 2).

We propose the designation *Candidatus* Dactylopiibacterium carminicum for the β-proteobacteria named here as END1, identified from the insects of all the species in the genus *Dactylopius*. This bacterium is thus far uncultured. It has unique sequences in the 16S rRNA gene at the following sites (homologs to *Escherichia coli* positions): (1) 69–96, GATTCAAGGGGCTTGCTCCTTGGGT, and (2) 461–476, GGTGAATATCCGAAGCC. The 16S rRNA gene has an average of G + C content of 54.76 mol%. *Dactylopiibacterium*: Dac.ty.lo.pi.i.bac.te'ri.um. N.L. n. *Dactylopius*, cochineal scientific genus name; L. neut. n. bacterium, N.L. neut. n. *Dactylopiibacterium*, a bacterium isolated from *Dactylopius* spp.; *caminicum*: car.mi.ni'cum. M.L. n. carminium, carmine; *carminicum*, belonging to carmine (red pigment) that is produced by all *Dactylopius* spp.

Clone O1 collected from *D. opuntiae* belongs to the order Rickettsiales. In this order, many intracellular symbionts and pathogens of eukaryotes have been found (Weinert et al. 2009). The sequence from O1 and other related sequences, however, did not group with any of the major clades of Rickettsia, meaning that they could represent new taxa within this group. In contrast to published data (Pankewitz et al. 2007), we did not find *Wolbachia* in *Dactylopius* spp.

The sequences from the clones close to free-living bacteria belong to soil or to plant associated bacteria. The location of some of these bacteria could be the gut, in which case its origin could plausibly be the sap that serves as food for the insects. It would be of ecological interest to explore this fact by analyzing the endophytic bacteria from the plants parasited by the insects. Their location inside the insect must be also determined.

Our description of *Dactylopius* spp. endosymbionts will be the basis for studying its role in the development and physiology of the insect. These bacteria could be implicated in providing amino acids, vitamins, or antimicrobial compounds to the host or in degrading plant toxic compounds, as occurs in other sap feeding insects.

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References Cited

Baumann, P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu. Rev. Microbiol. 59: 155–189.

Chávez-Moreno, C. K., A. Tecante, and A. Casas. 2009. The *Opuntia* (Cactaceae) and *Dactylopius* (Hemiptera: Dactylopiidae) in Mexico: a historical perspective of use, interaction and distribution. Biodivers. Conserv. 18: 3337–3355.

Cook, L. G., and P. J. Gullan. 2004. The gall-inducing habit has evolved multiple times among the eriococcid scale insects (Sternorrhyncha: Coccoidea: Eriococcidae). Biol. J. Linn. Soc. Lond. 83: 441–452.

Cook, L. G., P. J. Gullan, and H. E. Trueman. 2002. A preliminary phylogeny of the scale insects (Hemiptera: Sternorrhyncha: Coccoidea) based on nuclear small subunit ribosomal DNA. Mol. Phylogenet. Evol. 25: 43–52.

De Lotto, G. 1974. On the state and identity of the cochineal insects (Homoptera: Coccoidea: Dactylopiidae). J. Entomol. Soc. South Afr. 37: 167–193.

Eisner, T., R. Ziegler, J. L. McCormick, M. Eisner, E. R. Hoebeke, and J. Meinwald. 1994. Defensive use of an acquired substance (carminic acid) by predaceous insect larvae. Experientia 50: 610–615.

Gruwell, M. E., G. E. Morse, and B. B. Normark. 2007. Phylogenetic congruence of armored scale insects (Hemiptera: Diaspididae) and their primary endosymbionts from the phylum Bacteroidetes. Mol. Phylogenet. Evol. 44: 267–280.

Hernández-Hernández, F., F. García Gil de Muñoz, I. Del Río Dueñas, and H. Lanz Mendoza. 2005. La cochinilla fina del nopal, colorante mexicano para el mundo. Ciencia 56: 78–86.

Huelsenbeck, J. P., and F. Ronquist. 2001. MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17: 754–755.

Mathenge, C. W., P. Holford, J.H. Hoffmann, R. Spooner-Hart, G.A.C. Beattie, and H. G. Zimmermann. 2009. The biology of *Dactylopius tomentosus* (Hemiptera: Dactylopiidae). Br. Entomol. Res. 99: 551–559.

Moran, N. A. 2006. Symbiosis. Curr. Biol. 16: 866–871.

Moran, N. A., P. Tran, and N. M. Gerardo. 2005. Symbiosis and insect diversification: an ancient symbiont of sap-feeding insects from the bacterial phylum Bacteroidetes. Appl. Environ. Microbiol. 71: 8802–8810.

Moran, V. C., and H. G. Zimmermann. 1984. The biological control of cactus weeds: achievements and prospects. Biocontrol News Inform. 5: 297–320.

Noel, K. D., A. Sanchez, L. Fernandez, J. Leemans, and M. A. Cevallos. 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn5 insertions. J. Bacteriol. 158: 148–155.

Pankewitz, F., A. Zöllmer, M. Hilker, and Y. Gräser. 2007. Presence of *Wolbachia* in insect eggs containing antimicrobially active anthraquinones. Microb. Ecol. 54: 713–721.

Pérez-Guerra, G., and M. Kosztarab. 1992. Biosystematics of the family Dactylopiidae (Homoptera: Coccinea) with emphasis on the life cycle of *Dactylopius coccus* Costa. Studies on the morphology and systematics of scale insects. Bull. Virginia Agric. Exp. Station 92–1: 1–90.

Portillo, L. 2005. Origen de Costa *Dactylopius coccus* (Hemiptera: Dactylopiidae): ¿Norte o Sudamérica? Dugesiana 12: 1–8.

Portillo, M., and A. L. Vigueras. 2006. A review on the cochineal species in Mexico, hosts and natural enemies, pp. 249–256. *In* C. Mondragon Jacobo, G. Aranda Osorio, and W. B. Phippen (eds.), Vth International Congress on Cactus Pear and Cochineal. ISHS Acta Horticulturae, Chapingo, Mexico.

Rodríguez, L. C., M. A. Méndez, and H. M. Niemeyer. 2001. Direction of dispersion of cochineal (*Dactylopius coccus* Costa) within the Americas. Antiquity 75: 73–77.

Sambrook, J., and D. W. Russell. 2001. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Tautz, D., J. M. Hancock, D. A. Webb, C. Tautz, and G. A. Dover. 1988. Complete sequences of the rRNA genes of *Drosophila melanogaster*. Mol. Biol. Evol. 5: 366–376.

Thao, M. L., P. J. Gullan, and P. Baumann. 2002. Secondary (γ-Proteobacteria) endosymbionts infect the primary (β-Proteobacteria) endosymbionts of mealybugs multiple times and coevolve with their hosts. Appl. Environ. Micro. 68: 3190–3197.

Thao, M. L., L. Baumann, and P. Baumann. 2004. Organization of the mitochondrial genomes of whiteflies, aphids, and psyllids (Hemiptera, Sternorrhyncha). BMC Evol. Biol. 4: 25.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

von Dohlen, C. D., and N. A. Moran. 1995. Molecular phylogeny of the Homoptera: a paraphyletic taxon. J. Mol. Evol. 41: 211–223.

Wang, Y., U. Stingl, F. Anton-Erxleben, M. Zimmer, and A. Brune. 2004. '*Candidatus Hepatincola porcellionum*' gen. nov., sp. nov., a new, stalk-forming lineage of Rickettsiales colonizing the midgut glands of a terrestrial isopod. Arch. Microbiol. 181: 299–304.

Weinert, L. A., J. H. Werren, A. Aebi, G. N. Stone, and F. M. Jiggins. 2009. Evolution and diversity of *Rickettsia* bacteria. BMC Biol. 7: 6.

Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697–703.

Weon, H. Y., B. Y. Kim, S. H. Yoo, S. W. Kwon, S. J. Go, and E. Stackebrandt. 2008. *Uliginosibacterium gangwonense* gen. nov., sp. nov., isolated from a wetland, Yongneup, in Korea. Int. J. Syst. Evol. Microbiol. 58: 131–135.

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Species in *Wolbachia*? Proposal for the designation of '*Candidatus* Wolbachia bourtzisii', '*Candidatus* Wolbachia onchocercicola', '*Candidatus* Wolbachia blaxteri', '*Candidatus* Wolbachia brugii', '*Candidatus* Wolbachia taylori', '*Candidatus* Wolbachia collembolicola' and '*Candidatus* Wolbachia multihospitum' for the different species within *Wolbachia* supergroups

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ABSTRACT

Wolbachia are highly extended bacterial endosymbionts that infect arthropods and filarial nematodes and produce contrasting phenotypes on their hosts. *Wolbachia* taxonomy has been understudied. Currently, *Wolbachia* strains are classified into phylogenetic supergroups. Here we applied phylogenomic analyses to study *Wolbachia* evolutionary relationships and examined metrics derived from their genome sequences such as average nucleotide identity (ANI), *in silico* DNA–DNA hybridization (DDH), G + C content, and synteny to shed light on the taxonomy of these bacteria. Draft genome sequences of strains *w*DacA and *w*DacB obtained from the carmine cochineal insect *Dactylopius coccus* were included. Although all analyses indicated that each *Wolbachia* supergroup represents a distinct evolutionary lineage, we found that some of the analyzed supergroups showed enough internal heterogeneity to be considered as assemblages of more than one species. Thus, supergroups would represent supraspecific groupings. Consequently, *Wolbachia pipientis nomen* species would apply only to strains of supergroup B and we propose the designation of '*Candidatus* Wolbachia bourtzisii', '*Candidatus* Wolbachia onchocercicola', '*Candidatus* Wolbachia blaxterii', '*Candidatus* Wolbachia brugii', '*Candidatus* Wolbachia taylorii', '*Candidatus* Wolbachia collembolicola' and '*Candidatus* Wolbachia multihospitis' for other supergroups.

Introduction

Wolbachia is a genus of endosymbiotic bacteria that are wide spread in nature. *Wolbachia* endosymbionts do not have a free-living phase and are under confinement to particular hosts. It is estimated that *Wolbachia* may be found in 40% of arthropod species [106], while a previous report calculated 60% [44]. *Wolbachia* endosymbionts have been found associated with nematodes from the Onchocercidae family [22,54]. Interactions with their hosts range from parasitism to mutualism. In arthropods are mostly considered as parasites since they may manipulate host reproduction by mechanisms like parthenogenesis, feminization, male killing, and cytoplasmic incompatibility [12,80,100]. However, *Wolbachia* symbiosis has been implicated in host fitness [15,94], or as being necessary for oogenesis [25]; in nematodes they are regarded as mutualistic and essential for survival [58]. *Wolbachia* symbiosis is outstanding as it may cause host speciation events [11].

Some insects and their endosymbionts have a parallel evolutionary history, and cospeciation events have been described for both host and bacteria, especially primary endosymbionts [1,10,21,79,87]. For endosymbionts that have cospeciated with

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their hosts, endosymbionts in different hosts would be distinct species. It seems that cospeciation is rare in *Wolbachia* and insects as their phylogenies are usually not congruent [1,49,86,88]. Thus, adaptations to different hosts would not necessarily mean bacterial speciation. *Wolbachia* infections in insects may be recent in some cases [41,48], implying a short host–symbiont interaction that would not lead to speciation. Recent *Wolbachia* acquisitions may come from horizontal transfers from close or even distant insects [41]. In filarial nematode–*Wolbachia* associations, congruence between *Wolbachia* phylogenies and those of their host has been documented [9,17,32]. In this case, cospeciation between bacteria and their worm hosts seems to have occurred and a single origin of this symbiosis for supergroups C and D has even been proposed [32]. *Wolbachia* have become essential for nematode development and play an important role in host embryogenesis [58]. Nematodes treated with antibiotics cannot reach adulthood [13,93].

Wolbachia pipientis Hertig 1936 [43], was first observed in cells of the *Culex pipiens* mosquito [42]. Heterogeneity within *Wolbachia* has been revealed by sequence analysis of 16S rRNA genes and protein-coding genes, resulting in its distribution into sixteen phylogenetic supergroups, ten of which are found in arthropods (A, B, E, H, I, K, M, N, P, Q), five in nematodes (C, D, J, L, O) and one comprising both arthropod and nematode endosymbionts (F) [4]. The strains of *Wolbachia* detected in Australian spiders [81], were designed as Supergroup G but it was later revealed that it has a *wsp* gene that is a recombinant between those of A and B supergroups rather than being a distinct new supergroup [8]. A phylogenetic tree based on a multilocus analysis has been recently published giving insight about the relationships between *Wolbachia* supergroups [37]. A consensus of whether supergroups represent lineages of *W. pipientis* or distinct species has not been reached. Sequence divergence between supergroups seemed to indicate that each represented a species [78], however, other studies have indicated that they do not represent isolated genetic entities [7,99], as would be expected from *bona fide* species [61]. *Wolbachia* have been described as highly recombinogenic bacteria [6,7,99]. Multiple infections with different *Wolbachia* are frequent in the same insect individual [98,104], affording the opportunity for recombination between different strains, including not closely related ones [6,104]. Nevertheless, a recent study found that recombination is far higher within supergroups than between them [30]. Recombination events between supergroups are limited to small DNA fragments.

Endosymbiont confinement in a host leads to an inevitable dependence on the host. This is evident upon inspection of endosymbiont genomes, which generally lack many functions required for independent living. Classic taxonomy relying on phenotypic characterization of pure cultures as well as establishing genomic relatedness by DNA–DNA hybridization (DDH) experiments could not be applied to non-cultivable endosymbionts like *Wolbachia*. In the genomic era, however, metrics based on genome sequences like ANI (average nucleotide identity) and *in silico* DDH can be used as replacements for wet lab DDH [3,38], thus allowing the use of similar taxonomic criteria for both cultivable and non-cultivable prokaryotes. Furthermore, it is increasingly acknowledged that phenotypes should not be given as much importance for species delineation as they currently are [20,70,96].

Here, we evaluated the diversity of *Wolbachia* by performing phylogenomic analyses and by analyzing genome-derived metrics like ANI, *in silico* DDH, G+C content and synteny in order to shed light on the taxonomy of these endosymbionts. Additionally, we increased the genomic database of *Wolbachia* by reporting sequences from two strains recently obtained from the carmine cochineal insect *Dactylopius coccus*.

Materials and methods

Genome sequences

Sequences of all reported *Wolbachia* genomes were retrieved from GenBank database, except those of strains *w*Di and *w*Ls, which were available at http://nematodes.org/genomes/index_filaria.html [22]. Genomes of *Wolbachia* strains *w*DacA (Bioproject PRJNA274701) and *w*DacB (Bioproject PRJNA274698) were sequenced by a metagenomic approach from dissected cochineal insects of *Dactylopius coccus*. Detailed functional analyses of those genomic sequences will be reported elsewhere (Ramírez-Puebla et al., in preparation). For G+C content determination, contigs of each genome were concatenated, the number of G plus C nucleotides counted and the sum divided by the genome length. Genome of strain *w*Men was obtained from the *Strepsiptera* Genome Project [68], and genomes of strains *w*Fol, *w*Oc and *w*Cte were only deposited like Sequence Read Archive (SRA) so they were not included in G+C determination because they were not completely sequenced [36].

Phylogenomic analyses

Predicted proteomes were obtained from annotated genomes deposited at GenBank if available. The RAST server was also used for annotating and comparing whole genome sequences [5]. The AMPHORA2 pipeline [103], was used to identify a set of 31 conserved bacterial proteins from complete or draft genomes. Sequencing reads were obtained from the Sequence Read Archive (SRA) database to obtain phylogenetic markers for strains *w*Men, *w*Fol, *w*Oc, and *w*Cte. Reads were mapped against individual marker genes obtained from fully sequenced *Wolbachia* genomes using the runMapping option from Newbler (Roche). The obtained mapped reads were processed to obtain the markers for these strains by performing tblastn searches against reference protein sequences corresponding to the markers from other sequenced strains. Protein sequences were concatenated using the EMBOSS union web tool (http://emboss.bioinformatics.nl/cgi-bin/emboss/union). The concatenated sequences were then aligned using MUSCLE v.3.8.31 [29], and the resulting alignment was processed with Gblocks [18], to obtain conserved protein blocks and eliminate poorly aligned positions and divergent regions. The edited alignment contained 9151 amino acid positions. A maximum-likelihood analysis was then performed using the JTT substitution model under PhyML 3.0 [40]. Branch support values are based on 100 bootstrap replicates. The genomes from *Ehrlichia canis* (GenBank CP000107) and *Anaplasma marginale* (GenBank CP001079) were used as outgroups.

In silico *DDH and ANI calculations*

DDH estimates were computed using the Genome-to-Genome Distance Calculator version 2.0 [65], as recommended by Auch et al. [2,3], and Meier-Kolthoff et al. [65]. BLAST+ was used for alignment and formula 2 for genome distance estimation. ANI values were calculated as previously proposed [38], using the ANI calculator from the Kostas lab (http://enve-omics.ce.gatech.edu/ani/) with default parameters.

Synteny

Syntenic blocks between ten finished *Wolbachia* genomes were identified by BLASTN. Only blocks at least 3000 bp in length and with 80% identity or higher were used to construct the graphs using the Artemis comparison tool [16].

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Results and discussion

Genome-based relationships

Predicted evolutionary relationships between all 34 complete or almost complete *Wolbachia* genomes currently available and our two *Wolbachia* strains from *D. coccus*, *w*DacA and *w*DacB (Table 1) based on a set of conserved proteins is shown in Fig. 1. The distinctiveness of *Wolbachia* supergroups was evidenced by each forming a different and well-supported clade. The A and B supergroups clustered together in a branch separated from the C, D and F supergroups as previously observed by Nikoh et al. [69], for a set of six genomes using 52 ribosomal proteins. The phylogenetic reconstructions were also in agreement with a previous analysis obtained with a set of 90 orthologous genes from only eleven sequenced *Wolbachia* strains [22]. All the *Wolbachia* strains associated with *Culex* mosquitoes (*W. pipientis* representatives) tightly clustered in a single clade within supergroup B. The *Wolbachia* strain *w*Bol1-b from *Hypolimnas bolina* was also phylogenetically close to the *W. pipientis* strains. *Wolbachia* strains from *D. coccus* clustered into distinct supergroups. *w*DacA was resolved as the most phylogenetically distant strain within supergroup A, whereas *w*DacB was a member of supergroup B having *w*VitB from *Nasonia vitripennis* as its closest sequenced relative. The strain *w*Fol associated with the springtail *Folsomia candida* was found placed as the most distant of all the analyzed *Wolbachia* supergroups as reported previously [36].

G + C content

The G + C content of *Wolbachia* genomes ranged from 32.1% to 38.4% (Table 1) evidencing an enrichment of AT nucleotides as is common in endosymbionts [67]. Mean G + C contents of supergroups A, B, C and D were 35.5%, 34.0%, 32.4% and 33.4%, respectively. The sequenced representative of supergroup F had a G + C content of 36.3%. Although the analyzed genomes may not completely comprise the natural variation present in *Wolbachia* supergroups, it is worth noting that each supergroup seems to have a characteristic G + C content (Table 1).

In silico *DDH and ANI estimates*

DDH is the "gold standard" for species delineation in prokaryotes but it is not applicable for non-cultivable bacteria like *Wolbachia*. ANI represents a suitable surrogate for wet lab DDH as correlation analyses indicate that strains showing ANI higher or equal than 95–96% shared DDH values higher or equal than 70% and are thus considered to be of the same species [38]. Genome sequences also allow the estimation of *in silico* DDH values, and

Table 1
Characteristics of the sequenced *Wolbachia* genomes used in this work.

Wolbachia strain	Host species	GenBank accession number	Genome status	Number of contigs	Genome size (bp)	G + C%	Super group	Reference
*w*Mel	*Drosophila melanogaster*	AE017196	Complete	1	1,267,782	35.2	A	[102]
*w*MelPop	*Drosophila melanogaster*	AQQE00000000	WGS	80	1,239,155	35.1	A	[101]
*w*Ri	*Drosophila simulans* Riverside	CP001391	Complete	1	1,445,873	35.2	A	[52]
*w*Ha	*Drosophila simulans*	CP003884	Complete	1	1,295,804	35.1	A	[30]
*w*Sim	*Drosophila simulans*	AAGC00000000	WGS	629	1,063,100	35.4	A	[84]
*w*Au	*Drosophila simulans*	LK055284	Complete	1	1,268,461	35.2	A	[91]
*w*Rec	*Drosophila recens*	JQAM00000000	WGS	43	1,126,656	35.2	A	[66]
*w*Suzi	*Drosophila suzukii*	CAOU02000000	WGS	110	1,415,350	35.2	A	[89]
*w*Dwi	*Drosophila willistoni*	AAQP00000000	WGS	260	1,145,915	38.4	A	Remington et al. (unpublished)
*w*Ana	*Drosophila ananassae*	AAGB00000000	WGS	464	1,440,750	35.7	A	[84]
*w*Gmm	*Glossina morsitans morsitans*	AWUH00000000	WGS	241	1,019,510	35.2	A	[14]
*w*Uni	*Muscidifurax uniraptor*	ACFP00000000	WGS	256	867,873	35.2	A	[52]
*w*DacA	*Dactylopius coccus*	PRJNA274701	WGS	456	933,576	35.0	A	This study
*w*No	*Drosophila simulans*	CP003883	Complete	1	1,301,823	34.0	B	[30]
*w*Pip_Pel	*Culex quinquefasciatus*	AM999887	Complete	2	1,482,455	34.2	B	[51]
*w*Pip_JHB	*Culex quinquefasciatus*	ABZA00000000	WGS	21	1,543,661	34.2	B	[85]
*w*Pip_Mol	*Culex molestus*	HG428761	Complete	1	1,340,443	33.9	B	[74]
*w*Pip	*Culex pipiens molestus*	CACK00000000	WGS	888	1,479,531	34.3	B	Sinkins et al. (unpublished)
*w*Di	*Diaphorina citri*	AMZJ00000000	WGS	124	1,240,904	34.0	B	[83]
*w*Bol1-b	*Hypolimnas bolina*	CAOH00000000	WGS	144	1,377,933	33.9	B	[28]
*w*AlbB	*Aedes albopictus*	CAGB00000000	WGS	156	1,162,431	33.7	B	[63]
*w*DacB	*Dactylopius coccus*	PRJNA274698	WGS	321	1,282,277	34.2	B	This study
*w*VitB	*Nasonia vitripennis*	AERW00000000	WGS	523	1,107,643	33.9	B	[50]
*w*Cte	*Ctenocephalides felis*	SRR1222150	Raw data	–	–	–	B	[36]
*w*Oo	*Onchocerca ochengi*	HE660029	Complete	1	957,990	32.1	C	[24]
*w*Ov	*Onchocerca volvulus* strain Cameroon	HG810405	Complete	1	960,618	32.1	C	Cotton et al. (unpublished)
*w*Di	*Dirofilaria immitis*	PRJEB4154[a]	WGS	2	921,012	32.7	C	[22]
*w*Bm strain TRS	*Brugia malayi*	AE017321	Complete	1	1,080,084	34.2	D	[35]
*w*Bn	*Wuchereria bancrofti*	ADHD00000000	WGS	763	1,052,327	34.0	D	[26]
*w*Ls	*Litomosoides sigmodontis*	PRJEB4155[a]	WGS	10	1,048,936	32.1	D	[22]
*w*Fol	*Folsomia candida*	SRR1222159	Raw data	–	–	–	E	[36]
*w*Cle	*Cimex lectularius*	AP013028	Complete	1	1,250,060	36.3	F	[69]
*w*Oc	*Osmia caerulescens*	SRR1221705	Raw data	–	–	–	F	[36]
*w*Men	*Mengenilla moldrzyki*	SRX095325	WGS	–	–	–	F	[68]

[a] Accessions numbers correspond to the European Nucleotide Archive database as submitted by the original authors.

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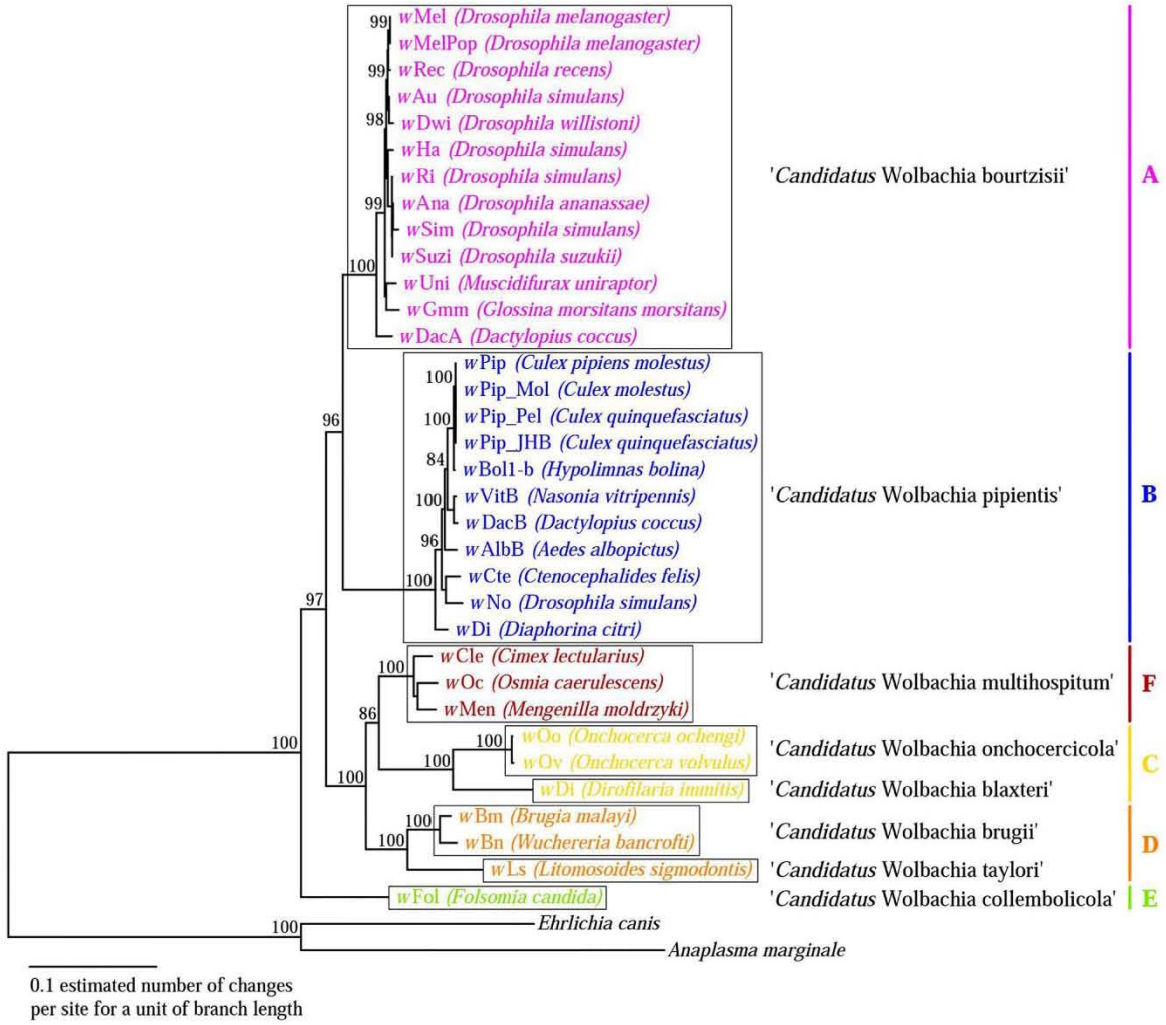

Fig. 1. Phylogenomic tree showing evolutionary relationships between *Wolbachia* strains inferred with PhyML based on a concatenated alignment of 31 marker proteins detected with AMPHORA2 and analyzed with the JTT substitution model. Hosts for each strain are indicated in forceps. Strains included in the new designations for each species name are boxed. Proposed species names are shown next to the boxes. Supergroups are shown to the right. Numbers at the branch points represent bootstrap support values based on 100 replicates. The scale bar represents the estimated number of amino acid changes per site for a unit of branch length.

these correlate very well with wet lab DDH [3]. Based in these criteria other bacteria as *Ensifer* and *Rhizobium* have been reclassified [71].

In silico DDH and ANI values were calculated for all pairs of analyzed *Wolbachia* genomes (Tables 2 and 3, respectively). Strains from different supergroups showed maximum ANI and *in silico* DDH values of 86.8% and 34.6%, respectively indicating that *Wolbachia* is comprised of several different species as previously suggested based on fewer ANI comparisons [75]. Within each supergroup, most members showed ANI values over 96% (Table 3) and *in silico* DDH values over 70% (Table 2), relatedness levels that are consistent with single species. However, in some supergroups there were strains with enough differences to put them below or near the borderline for species delineation. In supergroup A *Dactylopius* strain *w*DacA, and in supergroup B *Drosophila simulans* strain *w*No and *Diaphorina citri* strain *w*Di, showed *in silico* DDH values <62%, below the species circumscription level with all members of their own supergroups and ANI values just above of the species cut-off level (Table 2). In the phylogenomic analyses, strains *w*DacA, *w*No, *w*Di occupied peripheral positions within their supergroups (Fig. 1). *w*AlbB from *Aedes albopictus* and *w*DacB from *D. coccus* in supergroup B, also showed *in silico* DDH values below or very close to 70% with their supergroup neighbors, although these differences were not reflected by low ANI values (Table 2).

Within supergroup B, *in silico* DDH and ANI values were high among representative sequenced strains of *Wolbachia pipientis* (*w*Pip strains). Values were also high between *w*Bol1-b and *W. pipientis*. In contrast, comparisons between *w*Pip strains and other members of supergroup B produced values that were just over or slightly under the cutoff limits recommended for species delineation. The genome from strain *w*VitB had ANI value of around 97.4% when compared with the *w*Pip strain (Table 3) but most of their DDH estimates were below 70% (Table 2).

Clear examples of the existence of different species within supergroups were observed for nematode strains *w*Di of

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Table 2
In silico DNA–DNA hybridization (DDH).

in silico DNA-DNA[a] hybridization

Strain	Supergroup	w Mel	w MelPop	w Rec	w Au	w Dwi	w Uni	w Gmm	w Ana	w Ri	w Suzi	w Sim	w Ha	w DacA	w Pip	wPip_Mol	w Pip_Pel	w Pip_JHB	w BolI-b	w VitB	w DacB	w Di	w AlbB	w No	w Oo	w Ov	w Di	w Bm	w Bn	w Ls	w Cle
w Mel	A	100.0	97.7	89.3	93.9	91.5	75.8	71.2	72.8	71.9	72.1	73.1	73.2	57.6																	
w MelPop	A		100.0	95.3	93.6	92.6	76.2	71.7	72.8	72.1	72.3	74.9	73.2	57.6																	
w Rec	A			100.0	90.6	94.0	75.4	72.0	73.0	70.9	71.2	74.3	75.2	57.6																	
w Au	A				100.0	94.7	75.6	70.8	72.4	70.8	71.2	74.2	73.4	57.0																	
w Dwi	A					100.0	75.4	70.5	73.4	72.0	71.9	71.4	72.6	57.0																	
w Uni	A						100.0	74.5	73.5	73.7	73.8	73.3	73.6	58.6																	
w Gmm	A							100.0	67.1	65.1	66.1	67.9	69.5	56.4																	
w Ana	A								100.0	98.0	97.9	95.6	72.0	57.3																	
w Ri	A									100.0	98.6	85.2	69.2	55.9																	
w Suzi	A										100.0	90.7	69.5	55.9																	
w Sim	A											100.0	72.1	56.9																	
w Ha	A												100.0	56.7																	
w DacA	A													100.0																	
w Pip	B														100.0	100.0	96.7	96.4	83.1	71.5	69.5	58.8	63.7	57.3							
w Pip_Mol	B															100.0	84.4	87.6	81.5	70.8	68.5	58.2	63.0	56.6							
w Pip_Pel	B																100.0	86.1	78.0	68.4	67.5	56.7	61.9	55.2							
w Pip_JHB	B																	100.0	77.2	68.8	67.5	56.3	61.3	55.0							
w BolI-b	B																		100.0	69.8	68.0	58.1	61.9	57.6							
w VitB	B																			100.0	83.4	61.5	66.0	61.1							
w DacB	B																				100.0	59.6	62.6	58.6							
w Di	B																					100.0	59.0	54.5							
w AlbB	B																						100.0	58.9							
w No	B																							100.0							
w Oo	C																								100.0	96.1	29.2				
w Ov	C																									100.0	29.1				
w Di	C																										100.0				
w Bm	D																											100.0	69.8	36.5	
w Bn	D																												100.0	38.7	
w Ls	D																													100.0	
w Cle	F																														100.0

[a] Values <70% are shaded.

Table 3
Average nucleotide identity (ANI) percentage values between Wolbachia strains.

ANI[a] percentages for strain

Strain	Supergroup	w Mel	w MelPop	w Rec	w Au	w Dwi	w Uni	w Gmm	w Ana	w Ri	w Suzi	w Sim	w Ha	w DacA	w Pip	wPip_Mol	w Pip_Pel	w Pip_JHB	w BolI-b	w VitB	w DacB	w Di	w AlbB	w No	w Oo	w Ov	w Di	w Bm	w Bn	w Ls	w Cle
w Mel	A	100.0	100.0	99.6	99.6	99.4	97.9	97.3	97.4	97.8	97.7	97.9	97.7	94.0																	
w MelPop	A		100.0	99.6	99.6	99.4	97.9	97.4	97.5	97.8	97.7	97.8	97.6	94.9																	
w Rec	A			100.0	99.6	99.5	97.9	97.4	97.5	97.6	97.6	97.6	97.8	94.9																	
w Au	A				100.0	99.0	98.0	97.4	97.4	97.6	97.6	97.7	97.6	94.9																	
w Dwi	A					100.0	97.8	97.2	97.2	97.5	97.4	97.7	97.5	94.5																	
w Uni	A						100.0	97.6	97.7	97.6	97.6	97.6	97.5	95.0																	
w Gmm	A							100.0	96.9	96.9	96.9	96.9	97.0	94.6																	
w Ana	A								100.0	99.9	99.9	99.8	97.7	94.7																	
w Ri	A									100.0	100.0	99.9	97.7	94.7																	
w Suzi	A										100.0	99.9	97.7	94.7																	
w Sim	A											100.0	97.5	94.8																	
w Ha	A												100.0	94.8																	
w DacA	A													100.0																	
w Pip	B														100.0	100.0	99.9	99.9	99.1	97.4	97.3	95.8	96.3	95.3							
w Pip_Mol	B															100.0	99.8	99.8	99.0	97.2	97.2	95.7	96.1	95.2							
w Pip_Pel	B																100.0	99.9	98.9	97.2	97.1	95.8	96.1	95.1							
w Pip_JHB	B																	100.0	98.9	97.1	97.2	95.6	96.1	95.2							
w BolI-b	B																		100.0	97.2	97.1	95.7	96.2	95.3							
w VitB	B																			100.0	98.8	95.5	96.2	95.4							
w DacB	B																				100.0	95.5	96.1	95.4							
w Di	B																					100.0	95.4	94.8							
w AlbB	B																						100.0	95.3							
w No	B																							100.0							
w Oo	C																								100.0	99.4	86.1				
w Ov	C																									100.0	96.1				
w Di	C																										100.0				
w Bm	D																											100.0	96.3	85.8	
w Bn	D																												100.0	86.8	
w Ls	D																													100.0	
w Cle	F																														100.0

[a] Values <96% are shaded.

Dirofilaria immitis and *w*Ls in supergroups C and D, respectively (Tables 2 and 3). In these cases, both strains occupied peripheral positions within their supergroups (Fig. 1). Also, *in silico* DDH and ANI values supported the differentiation of these strains from other members of their respective supergroups supporting that they belong to different species.

Cospeciation between nematodes and *Wolbachia* is reflected in the fact that more distantly related nematodes [32], also have more distantly related *Wolbachia*. In supergroup D, *Wolbachia* from related nematode genera *Brugia* and *Wuchereria* are more closely related to each other than to *Wolbachia* from the more distant genus *Litomosoides* (Fig. 1, Tables 2 and 3). It is worth noting that *Wolbachia* infecting *Brugia* and *Wuchereria* are at the borderline for species definition by *in silico* DDH. Similarly, in supergroup C, *Wolbachia* from two species of *Onchocerca* are related but showed very low *in silico* DDH and ANI values to the endosymbiont of *D. immitis* (Fig. 1, Tables 2 and 3). Taken together, these data seem to indicate that each nematode genus harbors a different *Wolbachia* species. Nevertheless, the presence of supergroup F *Wolbachia* in both nematodes and insects, and its loss in some filarial species in

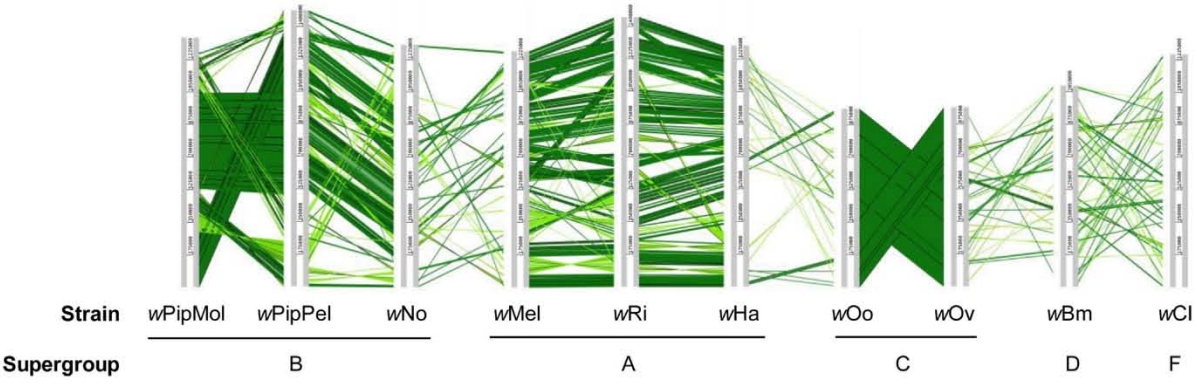

Fig. 2. Synteny between complete genomes of *Wolbachia* strains. Sequence blocks oriented in the same or inverted directions are shown in dark or light green, respectively. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

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the onchocercids, contravene the idea of cospeciation in general [33].

Genome synteny

Structural genome comparisons in other bacteria like rhizobia have shown that chromosome synteny is very well conserved within a species and less maintained between different species [39,77]. Synteny was used as a further criterion to distinguish *Wolbachia* species. It has been observed that levels of genome synteny are higher within than between *Wolbachia* supergroups [30], as it is evidenced in Fig. 2 for ten finished genomes. *Wolbachia* genomes have high levels of repetitive DNA and mobile genetic elements that lead to DNA rearrangements that diminish synteny even between related strains [30,35,52,102]. Genome rearrangements in other organisms represent recombination barriers and could lead to genetic isolation [76]. Strain *w*No showing significant divergence by *in silico* DDH and ANI values is less syntenic with its supergroup siblings. It would be worth investigating if a speciation process could start within a supergroup by *Wolbachia* strains developing novel genomic rearrangements, as discussed previously [30].

Conclusions

We showed here that *Wolbachia* supergroups represent distinct evolutionary lineages based on phylogenomics, G+C content, ANI, *in silico* DDH and synteny. Our results support the previous proposal that *Wolbachia* from different supergroups should be considered as genetically distinct clades not only from implications related to host confinement and their biology [72], but on the basis of molecular evidence [30,53,78]. Furthermore, we found heterogeneity within supergroups. The more divergent strains within each supergroup were recovered as outliers in the phylogenomic analyses. Not all strains, however, seem to have accumulated enough nucleotide sequence differences to show ANI values lower than 95–96%, used to delineate different species, with other distant strains in their supergroups (Table 3). Nevertheless, within a supergroup significant genome content differences were evidenced by low *in silico* DDH (less than 70%) and genome synteny among supergroup members was not always high. Thus, our novel analyses indicate that different species may occur inside a supergroup. Consequently, supergroups would have a supraspecific status.

Given the evidence reviewed and presented here, the name *W. pipientis* (Hertig 1936) [43], should be applied only to supergroup B strains. As *Wolbachia* are still uncultivable, the proper designation for supergroup B strains should be '*Candidatus* Wolbachia pipientis'. In order to distinguish the different species within *Wolbachia*, we propose the designation '*Candidatus* Wolbachia bourtzisii' for strains in supergroup A, '*Candidatus* Wolbachia onchocercicola' for endosymbionts of genus *Onchocerca* in supergroup C, '*Candidatus* Wolbachia blaxterii' for endosymbionts of *D. immitis* in supergroup C, '*Candidatus* Wolbachia brugii' for endosymbionts of nematodes from *Brugia* and *Wuchereria* species in supergroup D, '*Candidatus* Wolbachia taylorii' for endosymbionts of nematodes from *Litomosoides* species in supergroup D, '*Candidatus* Wolbachia collembola' for endosymbionts of Collembola arthropods in supergroup E and '*Candidatus* Wolbachia multihospitis' for *Wolbachia* strains hosted by nematodes and arthropods in supergroup F.

Description of '*Candidatus* Wolbachia bourtzisii'

'*Candidatus* Wolbachia bourtzisii' (bourt.zi'si.i. N.L. gen. n. *bourtzisii*, of Bourtzis, in honor of Kostas Bourtzis, as a recognition for his studies on *Wolbachia* and other bacteria associated with arthropods).

The description of the species '*Candidatus* Wolbachia bourtzisii' is based on the studies reported by Louis and Nigro [62], Sacchi et al. [82], Texeira et al. [94], and Zhukova and Kiseleva [105]. Cell size is 0.5 μm in *D. simulans*, and 0.5–1.0 μm in *D. melanogaster*. Cells are roundish and less frequently rod shaped and are surrounded by three enveloping membranes. The first is the plasmatic membrane and the second represents the outer part of the cell wall. The third one, closely related to the cytoplasm of the host cell, forms a vacuole for each single microorganism. Ribosomes and nucleic acid fibrils are observed in the cytoplasm. In *D. melanogaster* individual bacterial cells are distributed throughout the host cell cytoplasm, occasionally occurring as small groups. Bacteria occur in the ovarioles in high numbers and in germline cells like cytocytes, oogonia, oocytes and nurse cells.

The percentage of apoptotic cells in germaria are increased in *D. melanogaster* infected with *w*MelPop. Tetracycline treatments accelerated the time to death in *D. melanogaster* infected with *Drosophila* C virus (DCV) as the bacteria confer resistance to DCV by interfering with virus proliferation. The DNA G+C content is between 35.0 and 38.4 mol% as calculated from genomic sequences. Most strains exhibit a DNA G+C content of 35.2 mol%.

Description of '*Candidatus* Wolbachia onchocercicola'

'*Candidatus* Wolbachia onchocercicola' [on.cho.cer.ci'co.la. N.L. fem. n. Onchocerca a filarial nematode genus; L. suffix – *cola* (from L. masc. or fem. n. *incola*), a dweller, an inhabitant; N.L. fem. n. *onchocercicola*, a dweller of *Onchocerca*].

The description of the species '*Candidatus* Wolbachia onchocercicola' is based on the studies reported by Determan et al. [27], Egyed et al. [31], Horeauf et al. [46], Kozek and Marroquin [55], and Langworthy et al. [59]. Cell size is 0.3 up to 0.8 μm in diameter and 1.5 up to 1.8 μm in length. Cells are generally round or spherical shaped. Bacteria are located in the cytoplasm surrounded by a membrane-bound vacuole. In *Onchocerca lupi* each vacuole contains only one bacterium surrounded by a double membrane. In contrast, in *Onchocerca volvulus* they often form clusters and in *Onchocerca ochengi* some of them contain up to seven bacteria. Wolbachia live in the subcutaneous and connective tissues of their hosts, usually enclosed in fibrous cysts or nodules. In adults and larvae bacterial cells occur in the lateral cords, and in germinal tissues in females. Depletion of the endosymbiont by oxytetracycline in *O. ochengi* results in the death of adults and microfilaria. Also, there is a decline in the quantity of embryos and an increase in the proportion of embryos showing abnormal morphology. In *O. volvulus* doxycycline treatment blocks embryogenesis. The DNA G+C content is 32.1 mol% as calculated from genomic sequences.

Description of '*Candidatus* Wolbachia blaxteri'

'*Candidatus* Wolbachia blaxteri' (blax'ter.i. N.L. gen. n. *blaxteri*, of Blaxter, in honor of Mark Blaxter, in recognition of his molecular studies on nematodes and their associated *Wolbachia* symbionts).

The description of the species '*Candidatus* Wolbachia baxteri' is based on the studies reported by Kozek [55,56], McLaren et al., [64], and Sironi et al. [90]. Cell size is 0.3–1.0 μm in diameter and 4.5 μm in length. Cells are spherical or ovoid shaped. Bacteria are contained in an individual membrane-bounded host vacuole. Some bacterial cells have condensations of dense material within their cytoplasm. In *D. immitis* bacteria occur in the reproductive tract mainly in the ovary and the proximal region of the uterus, and are also found in oocytes and in all embryonic stages of microfilariae developing in the uterus. In lateral cords of adults, they occur as clusters that can

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fill most of the hypodermal tissue. Often they appear to surround the hypodermal nuclei. In embryos, five to ten bacteria per host cell are found. Also, bacteria are abundant in oogonia, eggs and early dividing embryos. Treatment with tetracycline blocks embryo development. The DNA G + C content is 32.7 mol% as calculated from genomic sequences.

Description of '*Candidatus* Wolbachia brugii'

'*Candidatus* Wolbachia brugii' (bru'gi.i. N.L. gen. n. *brugii*, of Brug, named after S. L. Brug, a Dutch parasitologist who first described the filarial nematode *Brugia malayi*, a model for the study of *Wolbachia*-nematode relationships).

The description of the species '*Candidatus* Wolbachia brugii' is based on the studies reported by Fischer et al. [34], and Landmann et al. [57,58], and Taylor et al. [92]. Cell size is 0.5 μm up to 1 μm. Cells are spherical or have an elongated shape and are surrounded with a double membrane. Bacteria are contained within membrane-bound vacuoles. In *Brugia malayi* clusters of bacteria are detected in microfilaria. In larvae L2, bacterial cells are detected in the hypodermis and in L3 and L4 larvae in the cells of lateral chord, in high numbers. In adult female worms, bacteria are commonly found in the lateral hypodermal cords, in hypodermis, and close to or inside the ovaries. Bacteria are also seen in the cells surrounding the basal lamina of the oviduct. In adult male worms, microfilariae, and third-stage larvae bacteria are detected in the lateral cord, but in lower numbers compared with females and dispersed in focal groups or as individual bacteria. They are also detected in testis and the border of vas deferens. In *Wuchereria bancrofti* bacteria show a similar distribution as in *B. malayi*, in small clusters or as a single bacterium.

Tetracycline treatments dramatically reduce the endosymbiont population in female adults of *B. malayi*. Pyknotic nuclei are observed throughout the ovaries and uteri in the female germline. Microfilaria resulting from a completed embryogenesis after antibiotic treatments, showed defects as abnormal muscle quadrants. Apoptotic nuclei are detected in the ovaries of treated females and become more numerous as the uteri is filled with embryos. The DNA G + C content is between 34.0 and 34.2 mol% as calculated from genomic sequences.

Description of '*Candidatus* Wolbachia taylori'

'*Candidatus* Wolbachia taylori' (tay'lo.ri. N.L. gen. n. *taylori*, of Taylor, in honor of Mark J. Taylor, in recognition of his studies on the role of *Wolbachia*–nematode symbiosis in human diseases and his search for treatments).

The description of the species '*Candidatus* Wolbachia taylori' is based on the studies reported by Chagas-Moutinho et al. [19], and Horeauf et al. [45]. Cell size is approximately1 μm and round shaped. Cells present a reduced cell wall and not a typical septum during cell division. Cells are surrounded by a host-derived vacuolar membrane. In *Litomosoides chagasfilhoi*, bacterial cells occur in regions of the hypoderm, in the oocytes, early-stage embryos and complete developed intrauterine microfilariae close to the cell host nucleus. In other filarial tissues, bacteria are found in intracellular vacuoles associated to the nuclear envelope. They are also observed in proximity to the endoplasmic reticulum. TEM suggested a single bacterium per vacuole.

Depletion by tetracycline results in infertility by blocking female worm development and early embryogenesis in *Litomosoides sigmodontis*. The DNA G + C content is 32.1 mol% as calculated from genomic sequences.

Description of '*Candidatus* Wolbachia collembolicola'

'*Candidatus* Wolbachia collembolicola' [col.lem.bo.li'co.la. N.L. n. pl. *Collembola* a lineage of hexapods; L. suffix – *cola* (from L. masc. or fem. n. *incola*), a dweller, an inhabitant; N.L. fem. n. *collembolicola*, a dweller of *Collembola*].

The description of the species '*Candidatus* Wolbachia collembolicola' is based on the studies reported by Czarnetzki and Tebbe [23], Pike and Kingcombe [73], Timmermans and Ellers [95], and Vandekerckhove et al. [97]. Cells detected in hexapod species of the order Collembola. Cell size is 0.2 μm up to 1.4 μm. Cells are pleomorphic from curved to almost hairpin-shaped. Cell wall lacks detectable peptidoglycan layer. Periplasmic space is of around 5–15 nm. Cells are surrounded by a host-derived vacuolar membrane. DNA filaments are visible in a rather diffuse network dispersed throughout the cell and interspersed with ribosomes. Cells occur in aggregations and are found mostly in close association with the rough endoplasmic reticulum in the ovaries. Fat bodies and interstitial cells as detected by TEM techniques or restricted to the ovary and brain as detected by FISH techniques.

Infection is obligatory for host offspring survival. The endosymbiont is sensitive to high-dose of rifampicin and heat treatments. High-dose tetracycline treatment is inefficient for removing cell infections. Bacteria obligate role early in the parthenogenetic developmental process includes egg hatching.

Description of '*Candidatus* Wolbachia multihospitum'

'*Candidatus* Wolbachia multihospitis' (mul.ti.hos'pi.tum. L. adj. *multus* many, numerous; L. n. *hospes -itis*, he who entertains a stranger, a host; N.L. gen. pl. n. multihospitum of numerous hosts, referring to the occurrence of the bacterium on various species of arthropods and nematodes).

The description of '*Candidatus* Wolbachia multihospitis' is based on the studies reported by Ferri et al. [33], Hosokawa et al. [47], and Lefoulon et al. [60]. In *Cimex lectularius* cell size is 0.5 up to 1.2 μm. Cells are rod-shaped. In males, bacterial cells are located in the testis-associated bacteriome, whereas in females they are located in bacteriomes and ovaries. Cells are also detected in the nutritive cord and developing oocytes. In the nematode *Madathamugadia hiepei*, they are detected in young and late embryos. In adult females they are observed in the ovaries and the intestinal wall. In contrast with other nematodes they are absent in the hypodermal lateral chord. In *Cercopithifilaria japonica* and *Mansonella perforata* bacteria are located in the epithelial somatic gonad and in the intestinal wall.

Elimination of the endosymbiont by rifampicin treatments in *C. lectularius* resulted in deformed developing eggs, reduction in the adult emergence rate and prolonged nymphal period. The DNA G + C content is 36.3 mol% as calculated from genomic sequences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2015.05.005

References

[1] Ahmed, M.Z., De Barro, P.J., Ren, S.X., Greeff, J.M., Qiu, B.L. (2013) Evidence for horizontal transmission of secondary endosymbionts in the *Bemisia tabaci* cryptic species complex. PLoS ONE 8, e53084.
[2] Auch, A.F., Klenk, H.P., Göker, M. (2010) Standard operating procedure for calculating genome-to-genome distances based on high-scoring segment pairs. Stand. Genomic Sci. 2, 142–148.
[3] Auch, A.F., von Jan, M., Klenk, H.P., Göker, M. (2010) Digital DNA–DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. Stand. Genomic Sci. 2, 117–134.
[4] Augustinos, A.A., Santos-García, D., Dionyssopoulou, E., Moreira, M., Papapanagiotou, A., Scarvelakis, M., Doudoumis, V., Ramos, S., Aguiar, A.F., Borges, P.A.V., Khadem, M., Latorre, A., Tsiamis, G., Bourtzis, K. (2011) Detection and characterization of *Wolbachia* infections in natural populations of aphids: is the hidden diversity fully unraveled? PLoS ONE 6, e28695.
[5] Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O. (2008) The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9, 75.
[6] Baldo, L., Lo, N., Werren, J.H. (2005) Mosaic nature of the *Wolbachia* surface protein. J. Bacteriol. 187, 5406–5418.
[7] Baldo, L., Bordenstein, S., Wernegreen, J.J., Werren, J.H. (2006) Widespread recombination throughout *Wolbachia* genomes. Mol. Biol. Evol. 23, 437–449.
[8] Baldo, L., Werren, J.L. (2007) Revisiting *Wolbachia* supergroup typing based on WSP: spurious lineages and discordance with MLST. Curr. Microbiol. 55, 81–87.
[9] Bandi, C., Anderson, T.J., Genchi, C., Blaxter, M.L. (1998) Phylogeny of *Wolbachia* in filarial nematodes. Proc. Biol. Sci. 265, 2407–2413.
[10] Baumann, P., Moran, N.A., Baumann, L. (2000) Bacteriocyte associated endosymbionts of insects. In: Dworkin, M. (Ed.), The prokaryotes, Springer, New York, pp. 155–189.
[11] Bordenstein, S.R. (2003) Symbiosis and the origin of species. In: Bourtzis, K., Miller, T.A. (Eds.), Insect Symbiosis, CRC Press, Boca Raton, pp. 283–304.
[12] Bordenstein, S.R., Paraskevopoulos, C., Dunning-Hotopp, J.C., Sapountzis, P., Lo, N., Bandi, C., Tettelin, H., Werren, J.H., Bourtzis, K. (2009) Parasitism and mutualism in *Wolbachia*: what the phylogenomic trees can and cannot say. Mol. Bio. Evol. 26, 231–241.
[13] Bosshardt, S.C., McCall, J.W., Coleman, S.U., Jones, K.L., Petit, T.A., Klei, T.R. (1993) Prophylactic activity of tetracycline against *Brugia pahangi* infection in jirds (*Meriones unguiculatus*). J. Parasitol. 79, 775–777.
[14] Brelsfoard., C., Tsiamis, G., Falchetto, M., Gomulski, L.M., Telleria, E., Alam, U., Doudoumis, V., Scolari, F., Benoit, J.B., Swain, M., Takac, P., Malacrida, A.R., Bourtzis, K., Aksoy, S. (2014) Presence of extensive *Wolbachia* symbiont insertions discovered in the genome of its host *Glossina morsitans morsitans*. PLoS Negl. Trop. Dis. 8, e2728.
[15] Brownlie, J.C., Cass, B.N., Riegler, M., Witsenburg, J.J., Iturbe-Ormaetxe, I., McGraw, E.A., O'Neill, S.L. (2009) Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. PLoS Pathog. 5, e1000368.
[16] Carver, T.J., Rutherford, K.M., Berriman, M., Rajandream, M.A., Barrell, B.G., Parkhill, J. (2005) ACT: the Artemis Comparison Tool. Bioinformatics 21, 3422–3423.
[17] Casiraghi, M., Bain, O., Guerrero, R., Martin, C., Pocacqua, V., Gardner, S.L., Franceschi, A., Bandi, C. (2004) Mapping the presence of *Wolbachia pipientis* on the phylogeny of filarial nematodes: evidence for symbiont loss during evolution. Int. J. Parasitol. 34, 191–203.
[18] Castresana, J. (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.
[19] Chagas-Moutinho, V.A., Silva, R., de Souza, W., Motta, M.C. (2015) Identification and ultrastructural characterization of the *Wolbachia* symbiont in *Litomosoides chagasfilhoi*. Parasit. Vector 8, 74.
[20] Chan, J.Z.-M., Halachev, M., Loman, N., Constantinidou, C., Pallen, M. (2012) Defining bacterial species in the genomic era: insights from the genus *Acinetobacter*. BMC Microbiol. 12, 302.
[21] Chen, X., Li, S., Aksoy, S. (1999) Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. J. Mol. Evol. 48, 49–58.
[22] Comandatore, F., Sassera, D., Montagna, M., Kumar, S., Koutsovoulos, G., Thomas, G., Repton, C., Babayan, S.A., Gray, N., Cordaux, R., Darby, A., Makepeace, B., Blaxter, M. (2013) Phylogenomics and analysis of shared genes suggest a single transition to mutualism in *Wolbachia* of nematodes. Genome Biol. Evol. 5, 1668–1674.
[23] Czarnetzki, A.B., Tebbe, C.C. (2004) Detection and phylogenetic analysis of *Wolbachia* in *Collembola*. Environ. Microbiol. 6, 35–44.
[24] Darby, A.C., Armstrong, S.D., Bah, G.S., Kaur, G., Hughes, M.A., Kay, S.M. (2012) Analysis of gene expression from the *Wolbachia* genome of a filarial nematode supports both metabolic and defensive roles within the symbiosis. Genome Res. 22, 2467–2477.
[25] Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M.E., Boulétreau, M. (2001) Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. Proc. Natl. Acad. Sci. 98, 6247–6252.
[26] Desjardins, C.A., Cerqueira, G.C., Goldberg, J.M., Dunning-Hotopp, J.C., Haas, B.J., Zucker, J., Ribeiro, J.M.C., Saif, S., Levin, J.Z., Fan, L., Zeng, Q., Russ, C., Wortman, J.R., Fink, D.L., Birren, B.W., Nutman, T.B. (2013) Genomics of *Loa loa*, a *Wolbachia*-free filarial parasite of humans. Nat. Genet. 45, 495–500.
[27] Determann, A., Mehlhorn, H., Ghaffar, F. (1997) Electron microscope observations on *Onchocerca ochengi* and *O. fasciata* (Nematoda: Filarioidea). Parasitol. Res. 83, 591–603.
[28] Duplouy, A., Iturbe-Ormaetxe, I., Beatson, S.A., Szubert, J.M., Brownlie, J.C., McMeniman, C.J., McGraw, E.A., Hurst, G.D.D., Charlat, S., O'Neill, S.L., Woolfit, M. (2013) Draft genome sequence of the male-killing *Wolbachia* strain *w*Bol1 reveals recent horizontal gene transfers from diverse sources. BMC Genomic 14, 20.
[29] Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.
[30] Ellegaard, K.M., Klasson, L., Näslund, K., Bourtzis, K., Andersson, S.G. (2013) Comparative genomics of *Wolbachia* and the bacterial species concept. PLoS Genet. 9, e1003381.
[31] Egyed, Z., Sréter, T., Széll, Z., Nyirő, G., Dobos-Kovács, M., Márialigeti, K., Varga, I. (2002) Electron microscopic and molecular identification of *Wolbachia* endosymbionts from *Onchocerca lupi*: implications for therapy. Vet. Parasitol. 106, 75–82.
[32] Fenn, K., Blaxter, M. (2004) Are filarial nematode *Wolbachia* obligate mutualist symbionts? Trends Ecol. Evol. 19, 163–166.
[33] Ferri, E., Bain, O., Barbuto, M., Martin, C., Lo, N., Uni, S., Landmann, F., Baccei, S.G., Guerrero, R., de Souza Lima, S., Bandi, C., Wanji, S., Diagne, M., Casiraghi, M. (2011) New insights into the evolution of *Wolbachia* infections in filarial nematodes inferred from a large range of screened species. PLoS ONE 6, e20843.
[34] Fischer, K., Beatty, W.L., Jiang, D., Weil, G.J., Fischer, P.U. (2011) Tissue and stage-specific distribution of *Wolbachia* in *Brugia malayi*. PLoS Negl. Trop. Dis. 5, e1174.
[35] Foster, J., Ganatra, M., Kamal, I., Ware, J., Makarova, K., Ivanova, N., Bhattacharyya, A., Kapatral, V., Kumar, S., Posfai, J., Vincze, T., Ingram, J., Moran, L., Lapidus, A., Omelchenko, M., Kyrpides, N., Ghedin, E., Wang, S., Goltsman, E., Joukoy, V., Ostrovskaya, O., Tsukerman, K., Mazur, M., Comb, D., Koonin, E., Slatko, B. (2005) The *Wolbachia* genome of *Brugia malayi*: endosymbiont evolution within a human pathogenic nematode. PLoS Biol. 3, e121.
[36] Gerth, M., Gansauge, M-T., Weigert, A., Bleidron, C. (2014) Phylogenomic analyses uncover origin and spread of the *Wolbachia* pandemic. Nat. Commun. 5, 5117.
[37] Glowska, E., Dragun-Damian, A., Dabert, M., Gerth, M. (2015) New *Wolbachia* supergroups detected in quill mites (Acari: Syringophilidae). Infect. Genet. Evol. 30, 140–146.
[38] Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P., Tiedje, J.M. (2007) DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. Int. J. Syst. Evol. Microbiol. 57, 81–91.
[39] Guerrero, G., Peralta, H., Aguilar, A., Díaz, R., Villalobos, M.A., Medrano-Soto, A., Mora, J. (2005) Evolutionary, structural and functional relationships revealed by comparative analysis of syntenic genes in Rhizobiales. BMC Evol. Biol. 55, 55.
[40] Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., Gascuel, O. (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59, 307–321.
[41] Heath, B.D., Butcher, R.D.J., Whitfield, W.G.F., Hubbard, S.F. (1999) Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. Curr. Biol. 9, 313–316.
[42] Hertig, M., Wolbach, S.B. (1924) Studies in *Rickettsia*-like micro-organisms in insects. J. Med. Res. 44, 329–374.
[43] Hertig, M. (1936) The rickettsia, *Wolbachia pipientis* (gen. et sp. n.) and associated inclusions of the mosquito, Culex pipiens. Parasitology 28, 453–486.
[44] Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., Werren, J.H. (2008) How many species are infected with *Wolbachia*? A statistical analysis of current data. FEMS Microbiol. Lett. 281, 215–220.
[45] Hoerauf, A., Volkmann, L., Nissen-Paehle, K., Schmetz, C., Autenrieth, I., Büttner, D.W., Fleischer, B. (2000) Targeting of *Wolbachia* endobacteria in *Litomosoides sigmodontis*: comparison of tetracyclines with chloramphenicol, macrolides and ciprofloxacin. Trop. Med. Int. Health 5, 275–279.
[46] Hoerauf, A., Mand, S., Adjei, O., Fleischer, B., Büttner, D.W. (2001) Depletion of *Wolbachia* endobacteria in *Onchocerca volvulus* by doxycycline and microfilariadermia after ivermectin treatment. The Lancet 357, 1415–1416.
[47] Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X-Y., Fukatsu, T. (2010) *Wolbachia* as a bacteriocyte-associated nutritional mutualist. PNAS 107, 769–774.
[48] Huigens, M.E., de Almeida, R.P., Boons, P.A.H., Luck, R.F., Stouthamer, R. (2004) Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. Proc. Biol. Sci. 271, 509–515.

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[49] Jäckel, R., Mora, D., Dobler, S. (2013) Evidence for selective sweeps by *Wolbachia* infections: phylogeny of *Altica* leaf beetles and their reproductive parasites. Mol. Ecol. 22, 4241–4255.
[50] Kent, B.N., Salichos, L., Gibbons, J.G., Rokas, A., Newton, I.L., Clark, M.E., Bordenstein, S.R. (2011) Complete bacteriophage transfer in a bacterial endosymbiont (*Wolbachia*) determined by targeted genome capture. Genome Biol. Evol. 3, 209–218.
[51] Klasson, L., Walker, T., Sebaihia, M., Sanders, M.J., Quail, M.A., Lord, A., Sanders, S., Earl, J., O'Neill, S.L., Thomson, N., Sinkins, S.P., Parkhill, J. (2008) Genome evolution of *Wolbachia* strain *w*Pip from the *Culex pipiens* group. Mol. Biol. Evol. 25, 1877–1887.
[52] Klasson, L., Westberg, J., Sapountzis, P., Näslund, K., Lutnaes, Y., Darby, A.C., Veneti, Z., Chen, L., Braig, H.R., Garrett, R., Bourtzis, K., Andersson, S.G.E. (2009) The mosaic genome structure of the *Wolbachia w*Ri strain infecting *Drosophila simulans*. Proc. Natl. Acad. Sci. U. S. A. 106, 5725–5730.
[53] Konstantinidis, K.T., Roselló-Móra, R. (2015) Classifying the uncultivated microbial majority: a place for metagenomics data in the *Candidatus* proposal. Syst. Appl. Microbiol., http://dx.doi.org/10.1016/j.syapm.2015.01.001
[54] Koutsovoulos, G., Makepeace, B., Tanya, V.N., Blaxter, M. (2014) Palaeosymbiosis revealed by genomic fossils of *Wolbachia* in a Strongyloidean nematode. PLoS Genet. 10, e1004397.
[55] Kozek, W.J., Marroquin, H.F. (1977) Intracytoplasmic bacteria in *Onchocerca volvulus*. Am J. Trop. Med. Hyg. 26, 663–678.
[56] Kozek, W.J. (2005) What is new in the *Wolbachia*/*Dirofilaria* interaction? Vet. Parasitol. 133, 127–132.
[57] Landmann, F., Voronin, D., Sullivan, W., Taylor, M.J. (2011) Anti-filarial activity of antibiotic therapy is due to extensive apoptosis after *Wolbachia* depletion from filarial nematodes. PLoS Pathog. 7, e1002351.
[58] Landmann, F., Foster, J.M., Michalski, M.L., Slatko, B.E., Sullivan, W. (2014) Co-evolution between an endosymbiont and its nematode host: *Wolbachia* asymmetric posterior localization and AP polarity establishment. PLoS Negl. Trop. Dis. 8, e3096.
[59] Langworthy, N.G., Renz, A., Mackenstedt, U., Henkle-du, K., Bronsvoort, M.B.C., Tanya, V.N., Donnelly, M.J., Trees, A.J. (2000) Macrofilaricidal activity of tetracycline against the filarial nematode *Onchocerca ochengi*: elimination of *Wolbachia* precedes worm death and suggests a dependent relationship. Proc. R. Soc. Lond. 267, 1063–1069.
[60] Lefoulon, E., Gavotte, L., Junker, K., Barbuto, M., Uni, S., Landmann, F., Laaksonen, S., Saari, S., Nikander, S., Souza, S.D., Casiraghi, M., Bain, O., Martin, C. (2012) A new type F *Wolbachia* from Splendidofilariinae (Onchocercidae) supports the recent emergence of this supergroup. Int. J. Parasitol. 42, 1025–1036.
[61] Lo, N., Paraskevopoulos, C., Bourtzis, K., O'Neill, S.L., Werren, J.H., Bordenstein, S.R., Bandi, C. (2007) Taxonomic status of the intracellular bacterium *Wolbachia pipientis*. Int. J. Syst. Evol. Microbiol. 57, 654–657.
[62] Louis, C., Nigro, L. (1989) Ultrastructural evidence of *Wolbachia* rickettsiales in *Drosophila simulans* and their relationships with unidirectional cross-incompatibility. J. Invertebr. Pathol. 24, 39–44.
[63] Mavingui, P., Valiente Moro, C., Tran-Van, V., Wisniewski-Dyé, F., Raquin, V., Minard, G., Tran, F.H., Voronin, D., Rouy, Z., Bustos, P., Lozano, L., Barbe, V., González, V. (2012) Whole-genome sequence of *Wolbachia* strain *w*AlbB, an endosymbiont of tiger mosquito vector *Aedes albopictus*. J. Bacteriol. 194, 1840.
[64] McLaren, D.J., Worms, M.J., Laurence, B.R., Simpson, M.G. (1975) Micro-organisms in filarial larvae (Nematoda). Trans. R. Soc. Trop. Med. Hyg. 69, 509–514.
[65] Meier-Kolthoff, J.P., Auch, A.F., Klenk, H.P., Göker, M. (2013) Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatic 14, 60.
[66] Metcalf, J.A., Jo, M., Bordenstein, S.R., Jaenike, J., Bordenstein, S.R. (2014) Recent genome reduction of *Wolbachia* in *Drosophila recens* targets phage WO and narrows candidates for reproductive parasitism. PeerJ 2, e529.
[67] Moran, N.A. (1996) Accelerated evolution and Muller's rachet in endosymbiotic bacteria. Proc. Natl. Acad. Sci. U. S. A. 93, 2873–2878.
[68] Niehuis, O., Hartig, G., Grath, S., Pohl, H., Lehmann, J., Tafer, H., Donath, A., Krauss, V., Eisenhardt, C., Hertel, J., Petersen, M., Mayer, C., Meusemann, K., Peters, R.S., Stadler, P.F., Beutel, R.G., Bornberg-Bauer, E., McKenna, D.D., Misof, B. (2012) Genomic and morphological evidence converge to resolve the enigma of *Strepsiptera*. Curr. Biol. 22, 1309–1313.
[69] Nikoh, N., Hosokawa, T., Moriyama, M., Oshima, K., Hattori, M., Fukatsu, T. (2014) Evolutionary origin of insect-*Wolbachia* nutritional mutualism. Proc. Natl. Acad. Sci. U. S. A. 111, 10257–10262.
[70] Ormeño-Orrillo, E., Martínez-Romero, E. (2013) Phenotypic tests in *Rhizobium* species description: an opinion and (a sympatric speciation) hypothesis. Syst. Appl. Microbiol. 36, 145–147.
[71] Ormeño-Orrillo, E., Servín-Garcidueñas, L.E., Rogel, M.A., González, V., Peralta, H., Mora, J., Martínez-Romero, J., Martínez-Romero, E. (2014) Taxonomy of rhizobia and agrobacteria from the Rhizobiaceae family in light of genomics. Syst. Appl. Microbiol. 38, 287–291.
[72] Pfarr, K., Foster, J., Slatko, B., Hoerauf, A., Eisen, J.A. (2007) On the taxonomic status of the intracellular bacterium *Wolbachia pipientis*: should this species name include the intracellular bacteria of filarial nematodes? Int. J. Syst. Evol. Microbiol. 57, 1677–1678.
[73] Pike, N., Kingcombe, R. (2009) Antibiotic treatment leads to the elimination of *Wolbachia* endosymbionts and sterility in the diplodiploid collembolan *Folsomia candida*. BMC Biol. 7, 54.
[74] Pinto, S.B., Stainton, K., Harris, S., Kambris, Z., Sutton, E.R., Bonsall, M.B., Parkhill, J., Sinkins, S.P. (2013) Transcriptional regulation of *Culex pipiens* mosquitoes by *Wolbachia* influences cytoplasmic incompatibility. PLoS Pathog. 9, e1003647.
[75] Richter, M., Roselló-Móra, R. (2009) Shifting the genomic gold standard for the prokaryotic species definition. Proc. Natl. Acad. Sci. U. S. A. 106, 19126–19131.
[76] Rieseberg, L.H. (2001) Chromosomal rearrangements and speciation. Trends Ecol. Evol. 16, 351–358.
[77] Rogel, M.A., Bustos, P., Santamaría, R.I., González, V., Romero, D., Cevallos, M.A., Lozano, L., Castro-Mondragón, J., Martínez-Romero, J., Ormeño-Orrillo, E., Martínez-Romero, E. (2014) Genomic basis of symbiovar mimosae in *Rhizobium etli*. BMC Genomic 15, 575.
[78] Ros, V.I., Fleming, V.M., Feil, E.J., Breeuwer, J.A. (2009) How diverse is the genus *Wolbachia*? Multiple-gene sequencing reveals a putatively new *Wolbachia* supergroup recovered from spider mites (Acari: Tetranychidae). App. Environ. Microbiol. 75, 1036–1043.
[79] Rosenblueth, M., Sayavedra, L., Sámano-Sánchez, H., Roth, A., Martínez-Romero, E. (2012) Evolutionary relationships of flavobacterial and enterobacterial endosymbionts with their scale insect hosts (Hemiptera: Coccoidea). J. Evol. Biol. 25, 2357–2368.
[80] Rousset, F., Bouchon, D., Pintureau, B., Juchault, P., Solignac, M. (1992) *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proc. R. Soc. Lond. B 250, 91–98.
[81] Rowley, S.M., Raven, R.J., McGraw, E.A. (2004) *Wolbachia pipientis* in Australian spiders. Curr. Microbiol. 49, 208–214.
[82] Sacchi, L., Genchi, M., Clementi, E., Negri, I., Alma, A., Ohler, S., Sassera, D., Bourtzis, K., Bandi, C. (2010) Bacteriocyte-like cells harbour *Wolbachia* in the ovary of *Drosophila melanogaster* (Insecta, Diptera) and *Zynginidia pullula* (Insecta, Hemiptera). Tissue Cell 42, 328–333.
[83] Saha, S., Hunter, W.B., Reese, J., Morgan, J.K., Marutani-Hert, M., Huang, H., Lindeberg, M. (2012) Survey of endosymbionts in the *Diaphorina citri* metagenome and assembly of a *Wolbachia w*Di draft genome. PLoS ONE 7, e50067.
[84] Salzberg, S.L., Dunning Hotopp, J.C., Delcher, A.L., Pop, M., Smith, D.R., Eisen, M.B., Nelson, W.C. (2005) Serendipitous discovery of *Wolbachia* genomes in multiple *Drosophila* species. Genome Biol. 6, R23.
[85] Salzberg, S.L., Puiu, D., Sommer, D.D., Nene, V., Lee, N.H. (2009) Genome sequence of the *Wolbachia* endosymbiont of *Culex quinquefasciatus* JHB. J. Bacteriol. 191, 1725.
[86] Schilthuizen, M., Stouthamer, R. (1997) Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. Proc. R. Soc. Lond. B 264, 361–366.
[87] Schröder, D., Deppisch, H., Obermayer, M., Krohne, G., Stackebrandt, E., Hölldobler, B., Goebel, W., Gross, R. (1996) Intracellular endosymbiotic bacteria of *Camponotus* species (carpenter ants): systematics, evolution and ultrastructural characterization. Mol. Microbiol. 21, 479–489.
[88] Shoemaker, D.D., Machado, C.A., Molbo, D., Werren, J.H., Windsor, D.M., Herre, E.A. (2002) The distribution of *Wolbachia* in fig wasps: correlations with host phylogeny, ecology and population structure. Proc. R. Soc. Lond. B 269, 2257–2267.
[89] Siozios, S., Cestaro, A., Kaur, R., Pertot, I., Rota-Stabelli, O., Anfora, G. (2013) Draft genome sequence of the *Wolbachia* endosymbiont of *Drosophila suzukii*. Genome Announc. 1, e00032–13.
[90] Sironi, M., Bandi, C., Sacchi, L., Di Sacco, B., Damiani, G., Genchi, C. (1995) Molecular evidence for a close relative of the arthropod endosymbiont *Wolbachia* in a filarial worm. Mol. Biochem. Parasitol. 74, 223–227.
[91] Sutton, E.R., Harris, S.R., Parkhill, J., Sinkins, S.P. (2014) Comparative genome analysis of *Wolbachia* strain *w*Au. BMC Genomic 15, 928.
[92] Taylor, M.J., Bilo, K., Cross, H.F., Archer, J.P., Underwood, A.P. (1999) 16S rDNA phylogeny and ultrastructural characterization of *Wolbachia* intracellular bacteria of the filarial nematodes *Brugia malayi*, *B. pahangi*, and *Wuchereria bancrofti*. Exp. Parasitol. 91, 356–361.
[93] Taylor, M.J., Bandi, C., Hoerauf, A.M., Lazdins, J. (2000) *Wolbachia* bacteria of filarial nematodes: a target for control? Parasitol. Today 16, 179–180.
[94] Teixeira, L., Ferreira, A., Ashburner, M. (2008) The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol. 6, e1000002.
[95] Timmermans, M.J.T.N., Ellers, J. (2009) *Wolbachia* endosymbiont is essential for egg hatching in a parthenogenetic arthropod. Evol. Ecol. 23, 931–942.
[96] Vandamme, P., Peeters, C. (2014) Time to revisit polyphasic taxonomy. Antonie van Leeuwenhoek 106, 57–65.
[97] Vandekerckhove, T.T., Watteyne, S., Willems, A., Swings, J.G., Mertens, J., Gillis, M. (1999) Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. FEMS Microbiol. Lett. 180, 279–286.
[98] Werren, J.H., Windsor, D., Guo, L.R. (1995) Distribution of *Wolbachia* among neotropical arthropods. Proc. R. Soc. Lond. B 262, 197–204.
[99] Werren, J.H., Bartos, J.D. (2001) Recombination in *Wolbachia*. Curr. Biol. 11, 431–435.
[100] Werren, J.H., Baldo, L., Clark, M.E. (2008) *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6, 741–751.
[101] Woolfit, M., Iturbe-Ormaetxe, I., Brownlie, J.C., Walker, T., Riegler, M., Seleznev, A., Popovici, J., Rancès, E., Wee, B.A., Pavlides, J., Sullivan, M.J., Beatson, S.A., Lane, A., Sidhu, M., McMeniman, C.J., McGraw, E.A., O'Neill, S.L. (2013)

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Genomic evolution of the pathogenic *Wolbachia* strain, *w*MelPop. Genome Biol. Evol. 5, 2189–2204.
[102] Wu, M., Sun, L.V., Vamathevan, J., Riegler, M., Deboy, R., Brownlie, J.C., McGraw, E.A., Martin, W., Esser, C., Ahmadinejad, N., Wiegand, C., Madupu, R., Beanan, M.J., Brinkac, L.M., Daugherty, S.C., Durkin, A.S., Kolonay, J.F., Nelson, W.C., Mohamoud, Y., Lee, P., Berry, K., Young, M.B., Utterback, T., Weidman, J., Nierman, W.C., Paulsen, I.T., Nelson, K.E., Tettelin, H., O'Neill, S.L., Eisen, J.A. (2004) Phylogenomics of the reprodutive parasite *Wolbachia pipientis* *w*Mel: a streamlined genome overrun by mobile genetic elements. PLoS Biol. 2, 327–341.
[103] Wu, M., Scott, A.J. (2012) Phylogenomic analysis of bacterial and archaeal sequences with AMPHORA2. Bioinformatics 28, 1033–10334.
[104] Yang, X.H., Zhu, D.H., Liu, Z., Zhao, L., Su, C.Y. (2013) High levels of multiple infections, recombination and horizontal transmission of *Wolbachia* in the *Andricus mukaigawae* (Hymenoptera; Cynipidae) communities. PLoS ONE 8, e78970.
[105] Zhukova, M., Kiseleva, E. (2012) The virulent *Wolbachia* strain *w*MelPop increases the frequency of apoptosis in the female germline cells of *Drosophila melanogaster*. BMC Microbiol. 12, S15.
[106] Zug, R., Hammerstein, P. (2012) Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS ONE 7, e38544.

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Two *Wolbachia* strains, *w*DacB and *w*DacA, from the metagenome of the cochineal insect *Dactylopius coccus* (Hemiptera: Dactylopiidae)

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ABSTRACT

Dactylopius species, known as cochineal insects, are the source of the carminic acid dye used worldwide. A *Dactylopius coccus* metagenome analysis revealed the presence of two *Wolbachia* strains, *w*DacA and *w*DacB, belonging to supergroups A and B, respectively. Draft genome sequences were recovered for both strains. Genome analysis indicated that the strains shared similar metabolic capabilities that are common to *Wolbachia*, including riboflavin, ubiquinone and haem biosynthesis but lacked other vitamin and cofactor biosynthesis as well as sugar uptake system, gluconeogenesis and non-oxidative pentose phosphate pathway. Genes for glycolysis were not found and there is a complete tricarboxylic acid cycle and limited amino acid biosynthesis. Uptake and catabolism of proline was evidenced in *Dactylopius Wolbachia* strains. Both strains possessed WO-like phage regions and type I and a type IV secretion systems. Several efflux systems found suggested the existence of metal toxicity within their host. Besides already described putative virulence factors like ankyrin domain proteins, VlrC homologues and patatin-like proteins, putative novel virulence factors related to those found in intracellular pathogens like *Legionella* and *Mycobacterium* are highlighted for the first time in *Wolbachia*. Candidate genes identified in other *Wolbachia* that are likely involved in cytoplasmic incompatibility were in *w*DacB but not in *w*DacA. Genome read coverage revealed that *w*DacB is more abundant in *D. coccus* than *w*DacA.

Keywords: Endosymbiont, *Wolbachia* genome, scale insect, double infection.

INTRODUCTION

Many insects have vertically transmitted symbiosis with bacteria that provide them with amino acids and vitamins (Moran, 2006). While most insect endosymbionts belong to the γ-Proteobacteria there are others in many other phyla (Moran et al., 2008). A remarkable case is *Wolbachia* endosymbiont that infect between 40 (Zug et al., 2012) and 66% (Hilgenboecker et al., 2008) of arthropod species. There are sixteen phylogenetic supergroups of *Wolbachia* identified and ten of them are associated to arthropods (Augustinos et al., 2011).

Wolbachia are phylogenetically affiliated to the α-Proteobacteria not far related to *Rickettsia*, *Ehrlichia* and *Anaplasma* (Williams et al., 2007). *Wolbachia* are nematode as well as arthropod symbionts (Hilgenboecker et al., 2008; Sommer & Streit, 2011) and have different effects in their hosts ranging from parasitism to mutualism with spatial and temporal spread of infections in some insect populations (Vavre & Charlat, 2012). In nematodes, *Wolbachia* provide vitamins, help to evade the host immune response and provide energy (Darby et al., 2012). In arthropods *Wolbachia* may alter the host reproduction by induction of parthenogenesis (Stouthamer et al., 1990), male killing, feminization (Stouthamer et al., 1999), and strain incompatibility (Rousset et al., 1992). On the other hand, *Wolbachia* may confer benefits to insects. While *Wolbachia* symbionts are capable to alter their reproduction causing sexual bias in the populations (Rousset et al., 1992) they may also play an important role in insect development and survival (Dedeine et al., 2001). These bacteria have been found infecting many tissues inside the insect body as reproductive tracts and somatic cells as bacteriocytes (Saha et al., 2012, Hosokawa et al., 2010). In *Nasoni*a wasps, *Wolbachia* cause male killing (Duplouy et al., 2013), but remotion of *Wolbachia* with antibiotics in *Asobara tabida* wasps inhibits maturation of oocytes (Dedeine et al., 2001). In *Drosophila* *Wolbachia* may confer protection against virus infections (Chrostek et al., 2013; Teixeira et al., 2008), and provide a fecundity benefit to females when subjected to low or high iron diets

(Brownlie et al., 2009). Thus, *Wolbachia* inside insects may be a facultative symbiont but can also be an obligate endosymbiont necessary for surviving (Dedeine et al., 2001).

There are eleven *Dactylopius* species (Ben Dov, 2006; Van Dam & May, 2012). Five of them are present in Mexico including the smallest and most distantly related *D. tomentosus* (Portillo & Vigueras 2006; Chávez-Moreno et al., 2009). *Dactylopius* insects feed exclusively on sap of cacti plants of genera *Opuntia* and *Nopalea* (Pérez-Guerra & Kostarab, 1992). Females of these scale insects spend all their lives on the host plant surface, whereas males are winged and short lived. These insects feed on a poor nutritional and low calorie diet, since cacti sap is mainly constituted by water (88-95%) and is low in nitrogen (0-0.5%) (Stintzing & Carle, 2005). The red pigment carmine is obtained from cochineal insects of the genus *Dactylopius*, especially from *D. coccus*, which is a domesticated species. Carmine has been used in Mexico for several hundred years, since prehispanic times. It is a natural dye used to color food, medicines, cosmetics, textiles and artworks and is considered safe for human consumption (Dapson, 2005), with antimicrobial and insecticidal properties (Eisner et al., 1980; Pankewitz et al., 2007). Currently, 300-350 tons are produced every year, being Peru and Spain the countries with the largest production (FAO bulletin).

Previously we described a β-Proteobacterium, *Candidatus* Dactylopiibacterium carminicum, and other diverse bacterial species associated with *Dactylopius* species present in Mexico (Ramírez-Puebla et al., 2010). Here, we extend the knowledge of *Dactylopius* endosymbionts and report the draft genome of two strains of *Wolbachia, w*DacA and *w*DacB.

MATERIALS AND METHODS.

Sample collection

D. coccus insects were provided by Campo Carmín Greenhouse (Morelos, Mexico). They were maintained on cacti plants (*Opuntia ficus-indica* var. Campo Carmín) in a growth room with controlled photoperiod (12L: 12D), temperature (25º C) and humidity (40-60%). Other *Dactylopius* species were collected from different states of Mexico: *D. confusus* from Tlaxcala, *D. ceylonicus* from state of México, *D. opuntiae* from Querétaro and Mexico city, and *D. tomentosus* from Hidalgo.

DNA extraction and detection of Wolbachia *in* D. coccus *individuals*

Metagenomic DNA was extracted from 2 g (20 individuals) of adult females which were superficially disinfected with ethanol (70%), rinsed with sterile distilled water and dissected with sterile forceps to remove exoeskeleton and guts. Cells in the hemolymph and debris were separated by centrifugation in Percoll gradient (adapted from Charles & Ishikawa, 1999). Percoll phases were observed under microscope and those with cells was selected for DNA extraction. DNA was extracted with DNeasy Blood & Tissue kit (QIAGEN) following the manufacturer instructions. Additionally, guts and ovaries from 40 *D. coccus* females were dissected using sterile forceps under a stereoscopic microscope. These organs were suspended in PBS and macerated using a sterile plastic pestle (Eppendorf). DNA was extracted as previously described and stored at -20ºC until use. DNA from the whole body of adult female from other *Dactytopius* species in Mexico (*D. confusus*, *D. ceylonicus*, *D. opuntiae*, and *D. tomentosus*) was also extracted.

PCR was performed using primers 27f (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492r (5'-TAC GGY TAC CTT GTT ACG ACT T-3') (Lane et al., 1991) targeted to gen 16S rRNA

and primers wsp81F (5'-TGG TCC AAT AAG TGA TGA AGA AAC-3') and wsp691R (5'-AAA AAT TAA ACG CTA CTC CA-3') targeted to gene *wsp* of *Wolbachia* (Braig et al. 1998).

High-throughput sequencing and assembly

Metagenomic DNA was sequenced with the following platforms: Illumina HiSeq 2000 platform (100 bp paired-end reads, 0.4 Kb insert size), Roche 454 (300 bp single-end reads) and PacBio SMRT cell technology (10.0 Kb CLR Library prep size). Sequencing was performed at Macrogen Inc. (Korea) for Illumina and 454 and Duke University Genome Sequencing Core (USA) for PacBio.

In a directed strategy to assemble *Wolbachia* genomes, Illumina and 454 reads were mapped to *Wolbachia* genomes using BLAST. The recovered reads were assembled using MetaVelvet Software (Namiki et al., 2012) with 95 k-mer length for Illumina. Contigs obtained were cut to 200bp to generate pseudoreads. Pseudoreads and reads from 454 ROCHE were assembled with Newbler software (Roche). For scaffolding SSPACE software (Boetzer et al., 2011) was used. Additionally the whole metagenome was assembled with SPAdes (Bankevich et al., 2012) using Illumina reads and PacBio filtered subreads, as well as 454 reads supplied to the assembler as non-trusted contigs.

Recovery of two Wolbachia strains from the* D. coccus *metagenome

Assembled contigs were aligned by Nucmer (Kurtz et al., 2004) against all available *Wolbachia* genomes and those producing significant matches (>50% coverage and >90% identity) were retained. Two contig groups were identified, one showing the highest similarity to sequences from supergroup A strains and other most similar to supergroup B strains. Those groups were independently used as seeds to capture additional contigs with the binning program Clams (Pati et

al., 2011) using tetranucleotide signatures for similarity matching. This process was recursively repeated until no additional contigs were recovered. All recovered contigs were aligned with BLASTX against the nr GenBank database to discard false positives, i.e. those contigs matching bacteria other than *Wolbachia*.

Annotation

The RAST server was used for gene prediction and annotation (Aziz et al., 2008). Manual curation of relevant genes was performed after comparisons with sequences deposited at the following databases: nr and Refseq via BLASTX (Benson et al., 2013), the Conserved Domain Database at GenBank (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), the Protein families (PFAM) database (Finn et al., 2014) and the Transport Classification Database (Saier et al., 2014).

Accession numbers

The genomes of *Wolbachia* strains *w*DacA and *w*DacB have been registered at GenBank and assigned BioProjects numbers PRJNA274701 and PRJNA274698, respectively.

RESULTS

Wolbachia in *Dactylopius*

During a metagenome analysis of the cochineal insect *D. coccus*, we observed that reads matching to *Wolbachia* sequences were the most abundant (data not shown). By recursively binning the assembled metagenome contigs looking for *Wolbachia* sequences, two bins were identified, one showing the highest similarity to sequences from *Wolbachia* supergroup A strains and other most similar to supergroup B strains. A phylogenomic analysis of those bins (Ramírez-Puebla et al., 2015), confirmed that they corresponded to two different *Wolbachia* strains belonging to supergroups A and B which will be referred here as *w*DacA and *w*DacB, respectively.

Wolbachia from supergroups A and B were previously reported in *Dactylopius* sp. collected in Lanzarote, Canary Islands, Spain (Pankewitz et al., 2007). When compared to the reported sequences from the Canarian *Wolbachia*, our Mexican *w*DacA and *w*DacB strains from *D. coccus* showed, respectively, 99.8% and 98.3% at the *ftsZ* gene, and 100% and 98.3 at the *wsp* gene. Thus, *Wolbachia* infecting *Dactylopius* sp. populations in the Canary Islands are closely related but distinct to the Mexican *Wolbachia*, being the divergence more pronounced among supergroup B representatives. Recently, a *Wolbachia* genome was recovered during a genome sequencing of *D. coccus* (Campana et al., 2015). The reported *w*Coc1 genome was found to belong to supergroup B by *ftsZ* gene sequence analysis but no analysis of the genome was provided. *w*Coc1 showed 92.4% and 98.2% ANI values with *w*DacA and *w*DacB, respectively, indicating that *w*Coc1 and *w*DacB belong to the same species. No further comparison against our strains was performed because the *w*Coc1 genome assembly was highly fragmented (1064 contigs, N50 size = 1387 bp).

Wolbachia PCR products were obtained from DNA extracted from other *Dactylopius* species collected in Mexico (*D. ceylonicus, D. opuntiae, D. confusus, and D. tomentosus*) (Data not

shown). These data show that *Wolbachia* might have started its endosymbiotic state before the genus *Dacytlopius* had diverged.

Genomes sequences of *Wolbachia* strains *w*DacA and *w*DacB

The number of contigs and N50 sizes of genome assemblies of *w*DacA and *w*DacB were 157 and 13.7 kb and 200 and 14.5 kb, respectively (Table 1), values which were above average in comparison to released WGS genomes of *Wolbachia* (data not shown). Read coverage were widely different between both genomes, 2700× in *w*DacB versus 174× in *w*DacA indicating that the former *Wolbachia* strain is predominant in the tissues of *D. coccus* used in this study. Detection of each *Wolbachia* strain by 16S gene PCR amplification and sequencing in isolated individuals of *D. coccus* seemed to corroborate that *w*DacB is more abundant than *w*DacA (Table 2). As other *Wolbachia* strains, *w*DacA and *w*DacB strains showed reduced genomes and low G+C contents (Table 1). Hypothetical genes represented 35% and 23% of the CDS genes in *w*DacA and *w*DacB, respectively. *Wolbachia* strains show high genome plasticity compared with other insect endosymbionts. The presence of a high proportion of mobile DNA and insertion sequences (Bordenstein and Reznikoff, 2005; Cordaux et al., 2008) may promote this plasticity. The two *Wolbachia* strains of *D. coccus* were not exceptions although it is worth mentioning that the genome of *w*DacB has a higher number of genes annotated as coding for mobile genetic elements and transposases (404, 24% of the CDS genes) in comparison to *w*DacA (120, 9% of the CDS genes).

Vitamin, coenzymes, cofactors and nucleotide synthesis

Both *Wolbachia* strain**s** from *D. coccu*s seemed able to synthesize riboflavin and ubiquinone (coenzyme Q). They also had genes required for purine and pyrimidine nucleotide biosynthesis.

They lacked complete biosynthesis genes for biotin, thiamine, coenzyme A, NAD and folic acid. Nevertheless, an uptake system for biotin and a gene for folate salvage were found encoded in each genome. Both strains also possessed a bacterioferritin gene and haem biosynthesis genes.

Metabolism

The set of genes for tricarboxylic cycle was complete in both genomes. There were genes for the pentose phosphate pathway but not the oxidative reactions. The phosphofructokinase gene is absent, suggesting that there may be gluconeogenesis but not glycolysis. A cytochrome c oxidase as well as components of the respiratory complex were found in both strains.

As has been observed in other *Wolbachia* and other Rickettsiales, most amino acid biosynthesis pathways were incomplete. However, catabolic genes for proline, aspartate, glutamate, and possibly cysteine were identified in both strains. Genes for glutamate dehydrogenase, glutamine synthetase (GS) and glutamate synthase (GOGAT) required for ammonia assimilation were also present. NifU was identified but no other nitrogen fixation genes. Nif proteins involved in the formation of FeS clusters or other metallo clusters can be found in organisms that do not fix nitrogen.

Complete set of genes for fatty acid biosynthesis were present in both genomes as well as for synthesis of the phospholipids phosphatidylethanolamine, phosphatidylglicerol and phosphatidylserine. No genes for lipopolysaccharide biosynthesis were found in either genome. *w*DacA and *w*DacB had genes for peptidoglycan synthesis but seemingly no genes for transpeptidases from chain-cross linking were found.

Transport

Both genomes encoded genes for ATP-binding cassette (ABC) transporters for uptake of phosphate (*pstABCS* genes), ferric iron, zinc, and possibly lipids; one for export of haem; and one gene for a Mg^{+2} (or Co^{+2}) transporter-E (MgtE) family importer. Several genes for putative amino acid symporters were shared by both genomes including five of the major facilitator superfamily (MFS), three of the alanine/glycine:cation symporter (AGCS) family, and one of the dicarboxylate/amino acid:cation symporter (DAACS) family. Strain *w*DacA but not *w*DacB had genes coding for an ABC uptake transporter for glutamine/glutamate. On the other hand, strain *w*DacB possessed three uptake systems of the drug/metabolite transporter (DMT) superfamily and two genes for organophosphate:phosphate MFS antiporters which were not present in *w*DacA. The former DMT transporters were >75% similar to the S-adenosylmethionine uptake transporter Sam of *R. prowazekii* (Tucker et al., 2003), and the highest similarities (~49%) of the latter MFS antiporters were to proteins of *R. prowazekii* which have been implicated in triose phosphate uptake used for phospholipid biosynthesis (Frohlich & Audia, 2013). No hexose transporter genes were found, supporting that there is no glycolysis in both strains.

Few export transporters were found in both genomes. Besides the haem exporter, both genomes encoded an ABC transporter putatively involved in organic solvent resistance, a CorC-family transporter for magnesium or cobalt efflux and a cation diffusion-facilitator (CDF) family exporter for zinc or cadmium. In addition, *w*DacA genome encoded two ABC superfamily transporters, one of the heavy metal transporter (HMT) family related to exporters for phytochelatins-Cd complexes and other of the multidrug resistance (MDR) family.

Secretion systems.

Of the two systems for protein export into the periplasm, only the general secretion *sec* system was found encoded in *w*DacA and *w*DacB genomes. Protein secretion into the extracellular environment is accomplish by several type of secretion systems of which only two were found in the *Wolbachia* strains of *Dactylopius*. Both genomes coded for the inner membrane component and the membrane fusion protein of a type I secretion system (T1SS) whose products were 95% and 83% identical, respectively, between the strains. The outer membrane TolC, a channel that acts in conjunction with the other T1SS components, was coded elsewhere in the genomes.

Both strains possessed one type IV secretion system (T4SS). The gene organization was similar to that found in other *Wolbachia* with two separated clusters, one including *virB3*, *virB4*, and four copies of *virB6*, and another cluster with *virB8*, *virB9*, *virB10*, *virB11* and *virD4*. As it is also observed in other *Wolbachia*, there were one paralogue of each *virB4*, *virB8* and *virB9* coded elsewhere in the genomes. Genes *virB1*, *virB2, virB5 and virB7* has been reported as being absent in *Wolbachia* and in Rickettsiales in general (Pichon et al., 2009). However, we found four and three homologues of the pilin *virB2* gene in *w*DacA and *w*DacB, respectively. The *virB2* homologues were not clustered with each other or with other *vir* genes. BLAST searches recovered *virB2* homologues in many *Wolbachia* genomes (data not shown) which are annotated mostly as hypothetical or membrane proteins.

In the symbiotic *w*Bm strain of the nematode *Brugia malayi*, the transcriptional regulators *w*BmxR1 and *w*BmxR2 bind to the promoter regions of some *vir* genes (Li and Carlow, 2012). *w*BmxR1 seems to regulate the *virB8* operon (which includes the upstream riboflavin biosynthesis gene *ribA*) and the second copy of *virB9*, while *w*BmxR2 controls the expression of the second copy of *virB4* (Li & Carlow, 2012). Homologues to *w*BmxR1 coding for proteins >74% identical

to the *w*BmxR1 product were found in both our *Wolbachia* strains, while a homologue to *w*BmxR2 was found only in *w*DacA (78% identity).

Stress response

Although living in a relatively protected environment inside their host cells, endosymbionts still retain genes required to cope with stressful conditions. Potassium homeostasis is important to react to changes in osmotic pressure and pH changes. One TrkG potassium uptake protein was found encoded in *w*DacA while there were two in *w*DacB. Both strains had a glutathione-regulated potassium-efflux system KefKL. An HtrA protease/chaperone for degradation of misfolded or mislocalized cell-envelope proteins was found encoded in each genome. Genes to contend with oxidative stress like those for a Fe superoxide dismutase, an alkyl hydroperoxide reductase, three glutaredoxins and for glutathione biosynthesis were also found. A single gene for a bacterial flavohemoglobin in each genome may be used to contend with nitrosative stress. Common proteins used for temperature stress such as DnaK-DnaJ-GrpE composing the DnaK chaperone system and GroEL-GroES composing the GroE chaperonin machinery were found encoded in both genomes as well as a CspA-family cold shock protein. A single sigma factor RpoH protein was encoded in each genome and may be used to stress response.

Virulence

Ankyrin domains are involved in protein-protein interactions and by interacting with specific regions of the host chromatin are able to modulate host gene transcription in other bacteria (Iturbe-ormaetxe et al., 2005). Genes coding proteins with ankyrin domains were found in both strain genomes although *wDacB* had double the number of genes in comparison to *w*DacA (34 versus 17 genes). Neither strain possessed genes for chemotaxis or motility via flagella or type IV pilus.

Two of the five MFS amino acid transporters of *w*DacA and *w*DacB belonged to the phagosomal nutrient transporter (Pht) family (Chen et al., 2008). A threonine-transporting member of this family is required for intracellular survival of *Legionella pneumophila* in macrophages (Sauer et al., 2005).

One gene in each *Wolbachia* strain coded for a protein bearing the mammalian cell entry (MCE) domain. Proteins in this family have been identified as necessary for intracellular colonization and survival by *Mycobacterium tuberculosis* and *Mycobacterium bovis* (Arruda et al., 1993; Flesselles et al., 1999).

Finally, genes coding for proteins which have been highlighted as candidates to induce cytoplasmic incompatibility were also found. Both genomes possessed two copies of the DNA-binding protein HU beta (Beckmann et al., 2013). *w*DacB but not *w*DacA had genes coding for proteins which are homologues to WPIP0282 that seems to be present only in *Wolbachia* strains inducing cytoplasmic incompatibility (Beckmann & Fallon, 2013). *w*DacB possessed two homologues of the transcriptional regulator *wtrM* gene whose product is able to upregulate the expression of a host gene implicated in cytoplasmic incompatibility (Pinto et al., 2013). The *wtrM* gene of *w*DacA was split in two halves by a frameshift mutation.

Phages

Several genes of gene clusters encoding phage proteins were found in both *Wolbachia* genomes. A complete cluster including phage head-baseplate or head-baseplate-tail genes was not recovered, clusters included either head, baseplate or tail genes. Paralogues genes located in different head or baseplate clusters in each genome suggested that each strain possesses more than one phage although it was not possible to determine if any of these phages is complete. The tail clusters, one

in each genome, were associated with putative virulence genes: two homologues of the VlrC protein in *w*DacA, and an ankyrin domain protein and a patatin-like protein in *w*DacB.

DISCUSSION

In this study we report on the presence of two *Wolbachia* strains, *w*DacA and *w*DacB, in Mexican individuals of the cochineal insect *D. coccus*. The big difference in read coverage between the genomes of *w*DacA and *w*DacB indicates that the latter strain is prevalent in *D. coccus*, at least in the tissue samples used here. Interestingly, *w*DacB but not *w*DacA possessed homologues coding for proteins which are likely candidates involved in causing cytoplasmic incompatibly, a mechanism promoting persistence and dissemination of *Wolbachia* in their hosts. In wasps double infections of supergroup A and group B strains have been found to influence reproductive and ecological isolation among sibling *Nasonia* species and therefore *Wolbachia* has been considered implicated in wasp speciation (Bordenstein et al., 2001).

Dactylopius insects feed on low-nutrient cacti sap and therefore have to develop strategies to acquire nutrients lacking in their diet from their symbiotic relationships with bacteria. In filarial nematodes, *Wolbachia* acts as an obligate symbiont. In other insects riboflavin is produced by their endosymbionts such as the gamma-Proteobacterium *Wigglesworthia* for the tse-tse flies (Akman et al., 2002) and *Buchnera* for the aphids (Nakabachi & Ishikawa, 1999) and is required for normal development in mosquitoes. It has been postulated that *Wolbachia* strains can also act as haem providers and/or helpers in maintaining iron homestossis in the host (Brownlie et al., 2009; Kremer et al., 2009). Both *Wolbachia* strains from *D. coccus* possessed genes for biosynthesis of riboflavin, nucleotides, haem, and the iron-storage protein bacterioferritin. Nevertheless, all these genes are common in the genomes of non-symbiotic sex-manipulator *Wolbachia* strains.

As has been observed in other *Wolbachia*, *w*DacA and *w*DacB do not have genes to produce most amino acids that their insect host requires, those may be provided by other bacteria present in *D. coccus* (Ramirez-Puebla et al., 2010) which could act as symbionts. Lacking most amino acid biosynthetic capabilities evidenced *Wolbachia* dependence on its host or on other endosymbionts. Retention of amino acid biosynthesis defines primary insect symbionts and its absence seems to be a characteristic of secondary symbionts (Darby et al., 2012). Lack of a functional glycolysis pathway and the presence of several amino acid uptake systems indicate that *Wolbachia* utilizes amino acid instead of sugars as nutrients. Many of the MFS transporters may be proline uptake systems which together with the presence of PutA for proline catabolism suggest that this amino acid could be a major nutrient for *Wolbachia*. In fact, high level expression of PutA has been demonstrated by proteomic analysis of *Wolbachia* (Baldridge et al., 2014). Interestingly, proline is an excellent precursor for riboflavin production in *Rhizobium* (Phillips et al., 1999).

Symbiotic and pathogenic bacteria can use effector proteins delivered to their host via the T4SS to promote intracellular colonization and persistence (Juhas *et al.*, 2008). T4SS is widely found in *Wolbachia* strains (Pichon *et al.*, 2009) and was also found in *w*DacA and *w*DacB. It was surprising to note that *virB2*, coding for the major T-pilus component, was reported as being absent in *Wolbachia* and other Rickettsiales (Pichon et al., 2009; Rancès et al., 2008). We found several *virB2* homologues in *w*DacA and *w*DacB as well as in many *Wolbachia* genomes. This is in agreement with recent data obtained in other Rickettsiales like *Anaplasma phagocytophilum* (Dugat et al., 2014) and *Neorickettsia risticii* (Lin et al., 2009) which do possesses several *virB2* paralogues. In *N. risticii*, VirB2 is located in the cell surface in agreement with its function as the major T4SS pilus protein (Lin et al., 2009). In *Anaplasma phagocytophilum*, the AnkA protein is exported via a T4SS (Lin *et al.*, 2007). Although T4SS are known to transport proteins and/or

DNA, an intriguing possibility is that they can act as nutrient transporters in *Wolbachia* given the scarcity of nutrient export systems in the genomes of these bacteria.

Several efflux systems for heavy metals were found in both genomes suggesting that *Wolbachia* from *D. coccus* have to cope with metal toxicity maybe contributed by their host diet. In this line, it is suggestive to note that the mucilage of *Opuntia* cacti act as a good water-soluble chelating polymer (polyelectrolyte) able to remove heavy metals from water (Barka et al., 2013) and that metal-bound phytochelatin can be found in *Opuntia* shoots (Landero Figueroa et al., 2007). Besides heavy metals, other harmful conditions are likely acting on *w*DacA and *w*DacB as both their genomes carried several genes to contend with abiotic stresses. Proteomic profiling of a mosquito *Wolbachia* strain has revealed a profile dominated by chaperones and stress proteins (Baldridge et al., 2014).

Another secretion system used by bacteria to interact with eukaryotes is the T1SS. In pathogenic bacteria, virulence factors such as haemolysins are secreted via this system. Secretion of some ankyrin domain proteins by T1SS has been reported in *Rickettsia* (Kaur et al., 2012) and *Ehrlichia* (Wakeel et al., 2011). Proteins bearing typical T1SS-secretion motifs could not be found in either of our *Wolbachia* genomes but it is worth noting that several ankyrin domain proteins were coded near the gene for the T1SS inner membrane component in *w*DacB.

Novel putative virulence factors related to those found in some intracellular pathogens like *Legionella* and *Mycobacterium* were found encoded in both genomes. The presence of these putative virulence factors has not been previously pointed out in *Wolbachia*.

Table 1. Genome features of the *Wolbachia* genomes from *D. coccus*

Feature	***w*DacA**	***w*DacB**
Number of Contigs	157	200
N50 (kb)	13,699	14,498
Estimaded genome size (bp)	1,170,639	1,509,974
G+C content (%)	35.1	34.1
CDS genes	1332	1695
With function	869	1307
Hypothetical	463	388
RNA genes		
rRNA	1	2
tRNA	31	39

Table 2. Abundance of *Wolbachia* strains in *D. coccus* individuals

	Number of 16S gene clones assigned to	
Individual	***w*DacA**	***w*DacB**
Female 1	0	15
Female 2	1	9
Female 3	1	6
Embryo 1	7	8
Embryo 2	2	11

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REFERENCES

[1] Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T., Hattori, M., and Aksoy, S. (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nat Genet* **32**, 402-7.

[2] Arruda, S., Bomfim, G., Knights, R., Huima-Byron, T., and Riley, L. W. (1993). Cloning of an *M. tuberculosis* DNA fragment associated with entry and survival inside cells. *Science* **261**, 1454-7.

[3] Augustinos, A.A., Santos-García, D., Dionyssopoulou, E., Moreira, M., Papapanagiotou, A., Scarvelakis, M., Doudoumis, V., Ramos, S., Aguiar, A.F., Borges, P.A.V., Khadem, M., Latorre, A., Tsiamis, G., Bourtzis, K. (2011) Detection and characterization of *Wolbachia* infections in natural populations of aphids: is the hidden diversity fully unraveled? PloS One 6, e28695.

[4] Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., and Zagnitko, O. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics* **9**, 75.

[5] Baldridge, G. D., Baldridge, A. S., Witthuhn, B. A., Higgins, L., Markowski, T. W., and Fallon, A. M. (2014). Proteomic profiling of a robust *Wolbachia* infection in an *Aedes albopictus* mosquito cell line. *Molecular Microbiology* **94**, 537-556.

[6] Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., and Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* **19**, 455-77.

[7] Barka, N., Abdennouri, M., El Makhfouk, M., and Qourzal, S. (2013). Biosorption characteristics of cadmium and lead onto eco-friendly dried cactus (*Opuntia ficus indica*) cladodes. *Journal of Environmental Chemical Engineering* **1**, 144-149.

[8] Beckmann, J. F., and Fallon, A. M. (2013). Detection of the *Wolbachia* protein WPIP0282 in mosquito spermathecae: Implications for cytoplasmic incompatibility. *Insect Biochemistry and Molecular Biology* **43**, 867-878.

[9] Beckmann, J. F., Markowski, T. W., Witthuhn, B. A., and Fallon, A. M. (2013). Detection of the *Wolbachia*-encoded DNA binding protein, HU beta, in mosquito gonads. *Insect Biochemistry and Molecular Biology* **43**, 272-279.

[10] Ben Dov, Y. (2006) *A systematic catalogue of eight scale insect families (Hemiptera: Coccoidea) of the world: Aclerdidae, Asterolecaniidae, Beesoniidae Carayonemidae, Conchaspididae, Dactylopiidae, Kerriidae and Lecanodiaspididae*. (388pp.) Elsevier Science Ltd.

[11] Benson, D. A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., and Sayers, E. W. (2013). GenBank. *Nucleic Acids Research* **41**, D36-D42.

[12] Boetzer, M., Henkel, C. V., Jansen, H. J., Butler, D., and Pirovano, W. (2011). Scaffolding pre-assembled contigs using SSPACE. *Bioinformatics* **27**, 578-9.

[13] Bordenstein, S. R., O'Hara, F. P., and Werren, J. H. (2001). *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature* **409**, 707-10.

[14] Bordenstein, S. R., and Reznikoff, W. S. (2005). Mobile DNA in obligate intracellular bacteria. *Nat Rev Micro* **3**, 688-699.

[15] Braig, H. R., Zhou, W., Dobson, S. L., Scott, L., & Neill, O. (1998). Cloning and Characterization of a Gene Encoding the Major Surface Protein of the Bacterial Endosymbiont *Wolbachia pipientis*. J Bacteriol. 180(9), 2373-2378.

[16] Brownlie, J. C., Cass, B. N., Riegler, M., Witsenburg, J. J., Iturbe-Ormaetxe, I., McGraw, E. A., and O'Neill, S. L. (2009). Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. *PLoS Pathog* **5**, e1000368.

[17] Campana, M. G., Robles García, N. M., and Tuross, N. (2015). America's red gold: multiple lineages of cultivated cochineal in Mexico. *Ecology and Evolution* **5**, 607-617.

[18] Chávez-Moreno, C. K., Tecante, A., Casas, A. (2009). The *Opuntia* (Cactaceae) and *Dactylopius* (Hemiptera: Dactylopiidae) in Mexico: a historical perspective of use, interaction and distribution. *Biodiversity and Conservation*, *18*(13), 3337–3355.

[19] Chen, D. E., Podell, S., Sauer, J.-D., Swanson, M. S., and Saier, M. H. (2008). The phagosomal nutrient transporter (Pht) family. *Microbiology* **154**, 42-53.

[20] Chrostek, E., Marialva, M. S. P., Esteves, S. S., Weinert, L. a, Martinez, J., Jiggins, F. M., & Teixeira, L. (2013). Wolbachia Variants Induce Differential Protection to Viruses in Drosophila melanogaster: A Phenotypic and Phylogenomic Analysis. *PLoS Genetics*, *9*(12), e1003896.

[21] Comandatore, F., Sassera, D., Montagna, M., Kumar, S., Koutsovoulos, G., Thomas, G., Repton, C., Babayan, S.A., Gray, N., Cordaux, R., Darby, A., Makepeace, B., Blaxter, M. (2013) Phylogenomics and analysis of shared genes suggest a single transition to mutualism in *Wolbachia* of nematodes. Genome Biol. Evol. 5, 1668–1674.

[22] Cordaux, R., Pichon, S., Ling, A., Pérez, P., Delaunay, C., Vavre, F., Bouchon, D., and Grève, P. (2008). Intense transpositional activity of insertion sequences in an ancient obligate endosymbiont. *Molecular Biology and Evolution* **25**, 1889-1896.

[23] Dapson, R. (2005). A method for determining identity and relative purity of carmine, carminic acid and aminocarminic acid. *Biotechnic & Histochemistry : Official Publication of the Biological Stain Commission*, *80*(5-6), 201–5.

[24] Darby, A.C., Armstrong, S.D., Bah, G.S., Kaur, G., Hughes, M.A., Kay, S.M. (2012) Analysis of gene expression from the *Wolbachia* genome of a filarial nematode supports both metabolic and defensive roles within the symbiosis. Genome Res. 22, 2467–2477.

[25] Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M. E., & Boulétreau, M. (2001). Removing symbiotic Wolbachia bacteria specifically inhibits oogenesis in a parasitic wasp. *Proceedings of the National Academy of Sciences*, *98*(11), 6247–6252.

[26] Dugat, T., Loux, V., Marthey, S., Moroldo, M., Lagree, A.-C., Boulouis, H.-J., Haddad, N., and Maillard, R. (2014). Comparative genomics of first available bovine *Anaplasma phagocytophilum* genome obtained with targeted sequence capture. *BMC Genomics* **15**, 973.

[27] Dunning Hotopp, J. C., Clark, M. E., Oliveira, D. C. S. G., Foster, J. M., Fischer, P., Muñoz Torres, M. C., … Werren, J. H. (2007). Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. *Science (New York, N.Y.)*, *317*(5845), 1753–6.

[28] Duplouy, A., Iturbe-Ormaetxe, I., Beatson, S.A., Szubert, J.M., Brownlie, J.C., McMeniman, C.J., McGraw, E.A., Hurst, G.D.D., Charlat, S., O'Neill, S.L., Woolfit, M. (2013) Draft genome sequence of the male-killing *Wolbachia* strain *w*Bol1 reveals recent horizontal gene transfers from diverse sources. BMC Genomics 14, 20.

[29] Eisner, T., Nowicki, S., Goetz, M., & Meinwald, J. (1980). Red Cochineal Dye (Carminic Acid): Its Role in Nature. *Science*, *208*(May), 1039–1042.

[30] Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Heger, A., Hetherington, K., Holm, L., Mistry, J., Sonnhammer, E. L. L., Tate, J., and Punta, M. (2014). Pfam: the protein families database. *Nucleic Acids Research* **42**, D222-D230.

[31] Flesselles, B., Anand, N. N., Remani, J., Loosmore, S. M., and Klein, M. H. (1999). Disruption of the mycobacterial cell entry gene of *Mycobacterium bovis* BCG results in a mutant that exhibits a reduced invasiveness for epithelial cells. *FEMS Microbiology Letters* **177**, 237-242.

[32] Frohlich, K. M., and Audia, J. P. (2013). Dual mechanisms of metabolite acquisition by the obligate intracytosolic pathogen *Rickettsia prowazekii* reveal novel aspects of triose phosphate transport. *Journal of Bacteriology* **195**, 3752-3760.

[33] Goto, S., Anbutsu, H., & Fukatsu, T. (2006). Asymmetrical interactions between Wolbachia and Spiroplasma endosymbionts coexisting in the same insect host. *Applied and Environmental Microbiology*, *72*(7), 4805–10.

[34] Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., & Werren, J. H. (2008). How many species are infected with Wolbachia ? A statistical analysis of current data. *FEMS Microbiology Letters*, *281*, 215–220.

[35] Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X-Y., Fukatsu, T. (2010) *Wolbachia* as a bacteriocyte-associated nutritional mutualist. PNAS. 107, 769–774.

[36] Huber, T., Faulkner, G., & Hugenholtz, P. (2004). Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics (Oxford, England)*, *20*(14), 2317–9.

[37] Iturbe-Ormaetxe, I., Burke, G. R., Riegler, M., O'Neill, S. L. (2005) Distribution, Expression, and motif variability of ankyrin domain genes in Wolbachia pipientis. J Bacteriol. 187(15), 5136-5145.

[38] Juhas, M., Crook, D. W. & Hood, D. W. (2008). Type IV secretion systems: tools of bacterial horizontal gene transfer and virulence. *Cell Microbiol* **10**, 2377-2386.

[39] June, M., Posada, D., Particular, F. O. R. A., See, P., & Public, G. (2005). Modeltest 3.7, *7*(June), 1–15.

[40] Kaur, S. J., Rahman, M. S., Ammerman, N. C., Beier-Sexton, M., Ceraul, S. M., Gillespie, J. J., and Azad, A. F. (2012). TolC-dependent secretion of an ankyrin repeat-containing protein of *Rickettsia typhi*. *J Bacteriol* **194**, 4920-32.

[41] Kent, B. N., Salichos, L., Gibbons, J. G., Rokas, A., Newton, I. L. G., Clark, M. E., & Bordenstein, S. R. (2011). Complete bacteriophage transfer in a bacterial endosymbiont (*Wolbachia*) determined by targeted genome capture. *Genome Biology and Evolution*, *3*(0), 209–18.

[42] Kremer, N., Voronin, D., Charif, D., Mavingui, P., Mollereau, B., and Vavre, F. (2009). *Wolbachia* interferes with ferritin expression and iron metabolism in insects. *PLoS Pathog* **5**, e1000630.

[43] Kumar, S., Tamura, K., & Nei, M. (1994). MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. *CABIOS*, *10*(2), 189–191.

[44] Landero Figueroa, J. A., Afton, S., Wrobelac, K., Wrobelac, K., and Caruso, J. A. (2007). Analysis of phytochelatins in nopal (*Opuntia ficus*): a metallomics approach in the soil–plant system. *J. Anal. At. Spectrom.* **22**, 897-904.

[45] Li, Z., and Carlow, C. K. S. (2012). Characterization of transcription factors that regulate the type IV secretion system and riboflavin biosynthesis in *Wolbachia* of *Brugia malayi*. *PLoS ONE* **7**, e51597.

[46] Lin, M., den Dulk-Ras, A., Hooykaas, P. J. & Rikihisa, Y. (2007). Anaplasma phagocytophilum AnkA secreted by type IV secretion system is tyrosine phosphorylated by Abl-1 to facilitate infection. *Cell Microbiol* **9**, 2644-2657.

[47] Lin, M., Zhang, C., Gibson, K., and Rikihisa, Y. (2009). Analysis of complete genome sequence of *Neorickettsia risticii*: causative agent of Potomac horse fever. *Nucleic Acids Res* **37**, 6076-91.

[48] Moran, N. A. (2006). Symbiosis. *Current Biology : CB*, *16*(20), R866–71.

[49] Moran, N. A, McCutcheon, J. P., & Nakabachi, A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, *42*, 165–90.

[50] Nakabachi, A., and Ishikawa, H. (1999). Provision of riboflavin to the host aphid, *Acyrthosiphon pisum*, by endosymbiotic bacteria, *Buchnera*. *J Insect Physiol* **45**, 1-6.

[51] Namiki, T., Hachiya, T., Tanaka, H., and Sakakibara, Y. (2012). MetaVelvet: an extension of Velvet assembler to de novo metagenome assembly from short sequence reads. *Nucleic Acids Res* **40**, e155.

[52] Nikoh, N., Tanaka, K., Shibata, F., Kondo, N., Hizume, M., Shimada, M., & Fukatsu, T. (2008). Wolbachia genome integrated in an insect chromosome: evolution and fate of laterally transferred endosymbiont genes. *Genome Research*, *18*(2), 272–80.

[53] Pankewitz, F., Zollmer, A., Hilker, M., and Graser, Y. (2007). Presence of *Wolbachia* in insect eggs containing antimicrobially active anthraquinones. *Microb Ecol* **54**, 713-21.

[54] Pati, A., Heath, L. S., Kyrpides, N. C., and Ivanova, N. (2011). ClaMS: A classifier for metagenomic sequences. *Stand Genomic Sci* **5**, 248-53.
[55] Pérez.Guerra, G., Kosztarab, M. (1992) Biosystematics of the family Dactylopiidae (Homoptera: Coccinea) with emphasis on the life cycle of Dactylopius coccus Costa. Bulletin No. 92-1. Virginia Agricultural Experiment Station, Virginia Polytechnic Institute and State University, Blacksburg, Virginia.
[56] Phillips, D. A., Joseph, C. M., Yang, G. P., Martinez-Romero, E., Sanborn, J. R., and Volpin, H. (1999). Identification of lumichrome as a *Sinorhizobium* enhancer of alfalfa root respiration and shoot growth. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 12275-80.
[57] Pichon, S., Bouchon, D., Cordaux, R., Chen, L., Garrett, R. A., and Grève, P. (2009). Conservation of the type IV secretion system throughout *Wolbachia* evolution. *Biochemical and Biophysical Research Communications* **385**, 557-562.
[58] Pinto, S. B., Stainton, K., Harris, S., Kambris, Z., Sutton, E. R., Bonsall, M. B., Parkhill, J., and Sinkins, S. P. (2013). Transcriptional regulation of *Culex pipiens* mosquitoes by *Wolbachia* influences cytoplasmic incompatibility. *PLoS Pathog* **9**, e1003647.
[59] Portillo, M. L., Vigueras, A. L. (2006). A Review on the Cochineal Species in Mexico, Hosts and Natural Enemies, 249–256.
[60] Ramirez-Puebla, S. T., Rosenblueth, M., Chavez-Moreno, C. K., de Lyra, M. C., Tecante, A., and Martinez-Romero, E. (2010). Molecular phylogeny of the genus *Dactylopius* (Hemiptera: Dactylopiidae) and identification of the symbiotic bacteria. *Environmental Entomology* **39**, 1178-83.
[61] Ramírez-puebla, S. T., Servín-Garcidueñas, L. E., Ormeño-Orillo, E., Vera-Ponce de León, A., Rosenblueth, M., Delaye, L., Martinez, J., Martinez-Romero, E. (2015) Species in *Wolbachia*? Proposal for the designation of '*Candidatus* Wolbachia bourtzisii', '*Candidatus* Wolbachia onchocercicola', '*Candidatus* Wolbachia blaxterii', '*Candidatus* Wolbachia brugii', '*Candidatus* Wolbachia taylorii', '*Candidatus* Wolbachia collembolicola' and '*Candidatus* Wolbachia multihospitis' for the different species within *Wolbachia* supergroups. Syst Appl Microbiol. In press.
[62] Rancès, E., Voronin, D., Tran-Van, V., and Mavingui, P. (2008). Genetic and functional characterization of the type IV secretion system in *Wolbachia*. *Journal of Bacteriology* **190**, 5020-5030.
[63] Rousset, F., Bouchon, D., Pintureau, B., Juchault, P., & Solignac, M. (1992). Wolbachia endosymbionts responsible for various alterations of sexuality in arthropods. *Proceedings. Biological Sciences / The Royal Society*, *250*(1328), 91–98.
[64] Saier, M. H., Reddy, V. S., Tamang, D. G., and Västermark, Å. (2014). The Transporter Classification Database. *Nucleic Acids Research* **42**, D251-D258.
[65] Sauer, J.-D., Bachman, M. A., and Swanson, M. S. (2005). The phagosomal transporter A couples threonine acquisition to differentiation and replication of *Legionella pneumophila* in macrophages. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 9924-9929.
[66] Sommer, R. J., & Streit, A. (2011). Comparative genetics and genomics of nematodes: genome structure, development, and lifestyle. *Annual Review of Genetics*, *45*, 1–20.
[67] Stintzing, F. C., & Carle, R. (2005). Cactus stems (Opuntia spp.): a review on their chemistry, technology, and uses. *Molecular Nutrition & Food Research*, *49*(2), 175–94.

[68] Stouthamer, R., Breeuwer, J. A. J., Hurst, G. D. D. (1999) *Wolbachia pipientis*: Microbial manipulator and arthropod reproduction. Annu. Rev. Microbiol. 53, 71-102.

[69] Teixeira, L., Ferreira, A., & Ashburner, M. (2008). The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. *PLoS Biology*, *6*(12), e2.

[70] Tucker, A. M., Winkler, H. H., Driskell, L. O., and Wood, D. O. (2003). S-adenosylmethionine transport in Rickettsia prowazekii. *J Bacteriol* **185**, 3031-5.

[71] Van Dam, A. R., & May, B. (2012). A new species of Dactylopius Costa (Dactylopius gracilipilus sp. nov.) (Hemiptera: Coccoidea: Dactylopiidae) from the Chihuahuan Desert, Texas, U.S.A. *Zootaxa*, *39*(3573), 33–39.

[72] Wakeel, A., den Dulk-Ras, A., Hooykaas, P. J., and McBride, J. W. (2011). *Ehrlichia chaffeensis* tandem repeat proteins and Ank200 are type 1 secretion system substrates related to the repeats-in-toxin exoprotein family. *Front Cell Infect Microbiol* **1**, 22.

[73] Weisburg, W. G., Barns, S. M., Pelletier, D. a, & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, *173*(2), 697–703.

[74] Wilkes, T. E., Darby, a C., Choi, J.-H., Colbourne, J. K., Werren, J. H., & Hurst, G. D. D. (2010). The draft genome sequence of Arsenophonus nasoniae, son-killer bacterium of Nasonia vitripennis, reveals genes associated with virulence and symbiosis. *Insect Molecular Biology*, *19 Suppl 1*, 59–73.

[75] Williams, K.P., Sobral, B. W., Dickerman, A. W. (2007) A robust species tree for the *Alphaproteobacteria*. J Bacteriol. 189 (13), 4578-4586.

[76] Zug, R., Hammerstein, P. (2012) Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS One 7, e38544.

DISCUSIÓN Y CONCLUSIONES

Las bacterias son el grupo de organismos más abundante en la Tierra y debido a que menos del 1% son cultivables en condiciones estándar de laboratorio los análisis de diversidad mediante enfoques independientes de cultivo son una poderosa herramienta que nos permite estudiar estas comunidades, entender los complejos procesos en los que participan y conocer sus interacciones con otros organismos dentro y fuera de la comunidad. Desde principios del siglo XX se han estudiado las interacciones entre insectos y bacterias (Buchner, 1965), pero los análisis se basaban en observaciones microscópicas meramente descriptivas. Hasta la fecha solamente algunos endosimbiontes como *Sodalis glossinidius*, endosimbionte de la mosca Tsetse (*Glossina morsitans morsintans*) (Dalet & Maudlin, 1999) y algunas cepas de *Wolbachia* (McMeniman et al., 2008), se han logrado cultivar en líneas celulares de mosquito bajo condiciones anoxigénicas.

La Biología molecular nos permite incrementar nuestro conocimiento sobre estas interacciones ya que ahora es posible conocer la identidad de estas bacterias, su historia evolutiva y su influencia en el insecto hospedero. Aunado a esto, el surgimiento de la Metagenómica ha mejorado las perspectivas ya que permite el análisis de comunidades aun más complejas. Gracias a estas aproximaciones logramos identificar las bacterias asociadas a *Dactylopius*, que son insectos de relevancia no sólo económica sino cultural en nuestro país. Entre las bacterias encontradas en las especies de *Dactylopius* presentes en México están algunas que comúnmente se asocian a plantas como *Acinetobacter*, *Herbaspirillum*, *Massilia*, *Mesorhizobium* y *Sphingomonas*, lo que podría indicar que se obtienen durante la alimentación en la savia del nopal, pero no queda claro si son residentes o sólo son transitorias, no obstante, se ha observado que microorganismos transitorios pueden contribuir a la homeostasis o ayudar a prevenir procesos inflamatorios en el intestino en otros animales (Plé et al., 2015) y que existe una comunidad variable dentro del

intestino que es específica de cada individuo y es retenida por periodos prolongados (Derrien & van Hylckama Vlieg, 2015), por lo que no se puede descartar la influencia de estas bacterias aun sin ser simbiontes obligados.

Una betaproteobacteria, se encontró en todas las especies analizadas, además de embriones y huevecillos lo que sugiere que es un simbionte en estos insectos. Se hizo un análisis parcial de esta bacteria a partir de las secuencias obtenidas en el metagenoma, pero se requieren más análisis para determinar con exactitud la influencia que tiene en su hospedero.

En el análisis metagenómico, la bacteria más abundante en *D. coccus* fue *Wolbachia* que, aunque ya se había reportando en estos insectos (Pankewitz et al., 2007), no se había analizado más detalladamente. En el primer análisis indepediente de cultivo por PCR no detectamos *Wolbachia*. Posteriormente nos percatamos de que los primers que utilizamos (FD1-RD1, Weisburg et al., 1991) no son complementarios con su secuencia del 16S rRNA. En otros estudios también se han observado sesgos debido a los primers usados por lo que los enfoques metagenómicos son más confiables para los estudios de diversidad.

Es de notar que la infección por *Wolbachia* en *D. coccus* se presenta como una infección múltiple de dos cepas, *w*DacA y *w*DacB, pertenecientes a los supergrupos A y B, respectivamente. En los escarabajos *Callosbruchus chinensis* se han observado infecciones múltiples por tres cepas de *Wolbachia* y cada una muestra diferentes patrones de colonización en tiempo y en el tejido que infecta (Ijichi et al., 2002). En avispas de la especie *Asobara tabida* también se observaron infecciones múltiples por tres cepas de *Wolbachia* y se determinó que entre más cepas infectan un individuo el costo fisiológico se incrementa, al parecer debido a la carga bacteriana; además observaron que la bacteria afecta la adecuación de su hospedero reduciendo la tasa de supervivencia y el peso (Mouton et al., 2004). En avispas del género *Nasonia* con infecciones múltiples de cepas de diferentes supergrupos se ha observado aislamiento reproductivo por lo que se propone que

Wolbachia puede influir en procesos de especiación (Bordenstein et al., 2001). También se encontraron genes en los genomas de estas cepas que codifican para proteínas asociadas con incompatibilidad citoplasmática. Para determinar si las cepas *w*DacA *y w*DacB de *D. coccus* también promueven estos efectos son necesarios más estudios.

A partir del ensamble de los genomas de las cepas de *Wolbachia* encontradas en *D. coccus* logramos hacer una reconstrucción filogenómica incluyendo genomas de Wobachias de otros supergrupos. Además, usamos parámetros como contenido de GC, Identidad promedio a nivel de nucleótidos (Average Nucleotide Identity, ANI), hibridación DNA-DNA (DDH) *in silico* y sintenia para separarlos en distintas especies, lo cual representa un gran avance en el estudio de *Wolbachia* ya que hasta ahora existe un conflicto en su clasificación dado que puede infectar nemátodos, distintos grupos de artrópodos y puede transferirse horizontalmente. Las filogenias basadas en el gen 16S rRNA, que usualmente se utiliza para reconstruir filogenias de bacterias, no pueden separar las cepas de manera confiable. Anteriormente ya se había separado *Wolbachia* en diferentes supergrupos y se había propuesto que cada uno deberían representar diferentes especies (Ros et al., 2009), pero nadie había realizado un análisis con todos los grupos para sustentar esta propuesta. Basándonos en nuestros análisis propusimos una clasificación de siete especies de *Wolbachia* y presentamos nombres para cada una.

Se ha observado que algunas cepas de *Wolbachia* como la cepa wMel-Pop de *Drosophila melanogaster* al ser transfectadas en células de mosquito interfieren con la proliferación del virus del dengue (DENV), el virus de Chikungunya y *Plasmodium* en *Aedes aegypti* (Iturbe-Ormaetxe et al., 2011) y además ocasiona incompatibilidad citoplasmática, por lo que actualmente existe interés de utilizar estas cepas como control de enfermedades, sin embargo debe tenerse cuidado con estos enfoques ya que no se conocen todavía los impactos ecológicos que implican. Mosquitos de la especie *Culex tarsalis*, infectados con *Wolbachia,* si bien son refractarios a la infección por

virus de dengue, parecen ser mas susceptibles a infecciones con el virus del Nilo (Dodson et al., 2014).

El papel de *Wolbachia* en nemátodos es claramente el de un simbionte que se ha considerado como una posible mitocondria que proporciona ATP al hospedero (Darby et al., 2012), pero su papel en insectos es diverso. En cultivos de células de insectos y en nemátodos se han estudiado los transcriptomas de *Wolbachia* en presencia y ausencia de wolbachias. En nemátodos se han encontrado muchos transcritos de wolbachias que codifican para ligandos de receptores Toll de mamíferos, que participan en inmunidad innata (Darby et al., 2012). Se ha dicho que las wolbachias son los simbiontes más abundantes de transmisión vertical y además son maestras en manipular la reproducción de sus hospederos, pero también se podría decir lo mismo sobre su capacidad de manipular la respuesta inmune de su hospedero. No es de extrañar que exista una enorme cantidad de publicaciones sobre este tema. Queda por entender el papel de wolbachias en *Dactylopius* y la principal limitante son los genes de función desconocida que hay en el genoma de estas bacterias.

A pesar de nuestra tendencia a estudiar los organismos como individuos aislados en lugar de partes de un sistema, cada vez se va reconociendo más la influencia de la simbiosis en la evolución de la vida en la Tierra. Todos los animales, no solamente los insectos, vivimos en un contexto de una microbiota residente por lo que nuestra supervivencia implica la transición de un metabolismo "egoísta" a uno más cooperativo (Douglas, 2012). La simbiosis es el ejemplo máximo del éxito mediante la colaboración y el poder de los beneficios que se originan a partir de las conexiones entre los seres vivientes (Relman, 2008). Después de todo, nuestras bacterias están aquí para recordarnos que nunca estamos solos.

APENDICE

Producto de esta investigación también se publicó un artículo en formato de minirevisión en el que se comparan los procesos de colonización de intestinos y raíces por bacterias simbiontes, resaltando las similitudes entre estos sistemas simbióticos.

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MINIREVIEW

Gut and Root Microbiota Commonalities

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Animal guts and plant roots have absorption roles for nutrient uptake and converge in harboring large, complex, and dynamic groups of microbes that participate in degradation or modification of nutrients and other substances. Gut and root bacteria regulate host gene expression, provide metabolic capabilities, essential nutrients, and protection against pathogens, and seem to share evolutionary trends.

Guts and roots are inhabited by many different bacteria (1–5), archaea (6–12), and viruses (13–16), as well as by eukaryotes (17–20), with some of them containing bacteria of their own (21–24). Variations in gut microbiota respond to age (25–28), diet (29–31), or species (32). Most insects have dozens of microbial species in their guts, while mammalian guts may contain thousands. Herbivores exhibit the largest diversity (32, 33), including probably plant-associated bacteria, especially endophytes (34) that, by being inside plant tissues, may survive stomach digestion. Transiting diet-borne bacteria may contribute to gut metabolic capacities. Different soil types, moisture (35), plant genotypes (36), age (37), and root lysates, secretions, or exudates (38) are determinants of root microbiotas. Factors that determine root exudates, such as availability of inorganic nutrients, temperature, light intensity, O_2/CO_2 level, or root damage, may indirectly affect root microbiotas (39). The presence of pathogens induces changes in microbiota composition in roots and guts (40, 41).

Guts and roots have large surface areas, with microvilli and folds or root hairs in some parts. Both roots and guts are structured, nonhomogenous habitats with pH, nutrient, water, and oxygen differential levels or gradients. Gradients would favor colonization by distinct bacteria that are more successful in some root or gut regions. In consequence, the multiple microhabitats that exist in roots and guts contribute to high species richness (42, 43). Different conditions are found in the cecum and distal colon in humans, with cecal and colon microbiotas containing a larger proportion of facultative anaerobes (44). Colon mucosal folds exhibit particular bacteria adapted to colonic conditions and maybe to mucin degradation (45). Some insects have specialized structures in their gut, such as midgut sacs and tubular outgrowths called ceca or crypts, in which they harbor specific bacteria (46), and others with less-complex guts also have pH and oxygen gradients in their guts (47). A steep oxygen gradient including an anaerobic root environment in water-saturated roots parallels the gut oxygen gradient and anaerobic gut systems. Clostridia, and especially members of the family *Ruminococcaceae*, are more prevalent than other anaerobes and methanogens, a trend which is similar in the different gut systems (48). These communities take care of the degradation of the complex organic matter in the outer root layers. Some gut and root acid-tolerant bacteria can modify their environment by lowering the pH when producing diverse acids (49, 50). Along the roots, there are physiological differences, and their exudates are secreted differentially at the apical meristem, root cap, or root hairs (42), creating different microhabitats. A single *Burkholderia* strain colonizes only discrete root regions (51), and different burkholderias were found at different soil depths (37).

"*Arabidopsis thaliana* root microbiome might assemble by core ecological principles similar to those shaping the mammalian microbiome in which core phylum level enterotypes provide broad metabolic potential combined with modest levels of host genotype-dependent associations" (35). Metacommunity theory may be applied to root microbiotas, as has been used to explain the assembly of the gut microbial community (52). Metacommunity theory is based on the concept of discontinuous patches and interactions that can satisfactorily describe bacterial patchy colonization of roots. Future applications of these concepts will assert their usefulness.

Remarkably, there are individual-to-individual variations in bacterial composition of the gut (2, 53) and roots (54). Individual differences may be due to genetic differences and stochastic colonization processes (52). Limited patterns (enterotypes) in relation to stratified variation were distinguished in human and insect gut microbiotas (2, 55); however, it is controversial if there are only a few enterotypes in humans or gradients of diversity (28). In plants, similar bacterial genera are recurrently isolated from rhizospheres (soil surrounding roots affected by plants) or roots (34, 56). In roots, *Rhizobium* strain diversity with functional differentiation is high (57). Strain variability in vitamin production has been detected among gut bifidobacteria (reviewed in reference 58). Similarly, lactobacilli (reviewed in reference 59) are a heterogeneous group of bacteria with partly probiotic character which have considerable variation in terms of molecular characteristics and preferred natural habitats.

With few exceptions (see below), the gut microbiota is different from that of other host organs, and similarly, the root microbiota shares only some bacteria with those of other plant organs.

ENVIRONMENTAL AND MATERNAL ACQUISITION

Root and gut microorganisms are usually acquired from the environment. Roots are colonized by bulk soil microorganisms at-

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tracted by chemotaxis and enriched by nutrients secreted by the roots in the rhizosphere. Animals also acquire their gut microbiota from their environment after they are born (60). In a few cases, microorganisms can be transferred vertically from mother to progenies. Endophytes present in plant seeds may subsequently colonize the roots and the rhizosphere. *Enterobacter asburiae*, found in maize kernels, is able to exit the roots and colonize the rhizosphere after the plant has established (61). Other seed bacteria do the same (54, 62). Animals can also acquire their gut microbiota from their mothers after being born, but there are cases of paternal transmission of symbionts, as in malaria vectors (63). Maternal transmission may occur before birth (64–66). When mammals are breast-fed, they acquire microorganisms that are present in the milk or on the mother's skin (67–69). Some stinkbug larvae acquire their mother's gut bacteria from contaminated eggs, by coprophagy, or by capsule-mediated transmission just after they have hatched (46). In view of the vertical and environmental transmission of root and gut microbes, gnotobiotic animals or plants are needed to clearly evaluate the effects of selected strains on hosts.

FUNCTIONAL REDUNDANCY AND ROLE OF MINORITIES

It seems that different microbiota composition may lead to the same and stable function. This may apply to gut and root bacteria and has been found to be true in methanogenic reactors (70). Similar degrading capacities are found in different gut bacteria (reviewed in reference 71). In roots, many different bacterial genera and species produce hormones, auxins, cytokinins, or gibberellins (reviewed in references 56 and 72). Our research group found that riboflavin is produced and excreted by different strains from several species of *Methylobacterium*, *Rhizobium*, *Sinorhizobium*, and *Bacillus*, both in rice and alfalfa root exudates and in pure cultures in minimal medium (our unpublished data). *In vitro* excretion of riboflavin by a large diversity of bacteria, including *Chromobacterium violaceum* and *Pantoea agglomerans*, was reported earlier (73), and both riboflavin and lumichrome (which is derived from riboflavin) stimulate root respiration (74). Additionally, many different plant-associated bacteria inhibit pathogenic fungi or bacteria (reviewed in reference 56).

Minority species present in the microbiota may help cover some of the host-specific needs. Methanogens, methylotrophs, and nitrogen-fixing bacteria are minor components in guts and rhizospheres (11, 75–78); however, they have important ecological roles. In some roots and guts, nitrogen fixation provides nitrogen to plants (79) and insects (80–82).

GUT AND ROOT BACTERIA ENHANCE THE METABOLIC CAPACITIES OF THEIR HOSTS

It is remarkable that gut bacteria are rich in sugar hydrolases (83) and other catabolic genes, such as those for tannin (84), cholesterol (85), or mucin (gut glycosylated proteins) (86). Similarly, capacities to degrade polyphenols, polysaccharides, protocatechuate, and proteins and to solubilize phosphate and weather rocks (50, 54, 87, 88) are prevalent among different rhizospheric bacteria. Mimosine-degrading bacteria are found in mimosa plants that produce mimosine (89), and cows that have such bacteria in their rumen are capable of degrading it (90). Alginate-degrading bacteria are found in abalone and human guts of algae consumers in Japan (91). The outstanding degrading capacities of root bacteria are the basis of rhizoremediation of polluting substances (92, 93) and are also evidenced in medical drug transformation or degradation in the human gut (94–96). Interestingly, in bioremediation, the abilities of bacteria to degrade soil pollutants may be triggered by flavonoids (97).

Gut and rhizospheric bacteria produce vitamins as riboflavin, as stated above. Vitamin B_{12} is an exclusive product of prokaryotes (98), and it is produced by plant root and gut bacteria (99–102). Essential amino acids and vitamins B and K are produced by gut bacteria (reviewed in reference 58). An alcohol dehydrogenase from the commensal bacterium *Acetobacter pomorum* modulates *Drosophila* developmental and metabolic homeostasis via insulin signaling (103). While root bacteria produce plant hormones that have effects on plant growth (reviewed in reference 56), gut bacteria seem to regulate animal behavior (104, 105).

GUT AND ROOT MICROBIOTAS COMPETE WITH PATHOGENS

Gut and root microbiotas suppress pathogens (reviewed in references 56 and 106). The human control of root bacteria has been envisaged as a manner to promote plant growth and health with benefits to agriculture (93, 107). Bacterial inoculants in agriculture and forestry are considered equivalent to probiotics (beneficial microbes provided as supplements) for animal health. Probiotics stimulate host defense systems and the competitive exclusion of pathogens, as plant growth-promoting rhizobacteria do (108). Seeds may harbor a reservoir of probiotics for their seedlings (54, 109). Prebiotics are added nutrients used to stimulate desirable bacteria in humans (110). We may even speculate that prebiotics were invented by roots, as some substances from their exudates stimulate bacterial growth selectively (89, 111, 112).

For over one hundred years, inoculants have been provided to plants in agricultural fields with variable success. Recently, a large number of commercial products whose effects are not always desirable have appeared to promote plant growth. Similarly, an increased number of probiotics and prebiotics whose effects have not been completely evaluated in different human populations are coming to the market. Gut gene expression in response to probiotics varies from person to person (113). In many cases, clinical benefits have been obtained in patients with specific probiotic strains (114).

Experience with plants has shown that appropriate use and regulation of probiotics (inoculants) is difficult to achieve. Undesirable genetic characteristics, such as denitrifying capacities, have been identified among inoculants (115). Strains used as probiotics should not contain glucosaminidase or glucuronidase genes that seem to have roles in producing toxic substances in the gut (reviewed in reference 116), but these recommendations may not be easily followed.

SIMILAR BACTERIUM-HOST INTERACTIONS IN GUTS AND ROOTS

Differential gene expression of bacteria in hosts. Bacterium-plant interactions have been studied for many years, and a molecular ping pong between rhizobia and plants that may serve as a model to analyze insect or human gut symbioses is known (reviewed in references 1 and 117). In rhizobium-plant molecular dialogue, *Rhizobium* NodD receptors, which bind root exudate molecules, function as transcriptional regulators that induce the expression of several genes, including *nod* genes and secretion systems (reviewed in references 117 and 118). Extrusion pumps are inducible by flavonoids that are present in root exudates but

do not require NodD genes (119). Many ABC transporter systems are induced by the respective substrate or other molecules from roots (111, 120).

In roots, bacteria have a differential gene expression that supposedly allows them to adapt to the root environment. Genes involved in root exudate usage, root attachment, and survival are induced in bacteria colonizing roots (120, 121). *In vitro* expression technology (IVET) (122), proteomic analysis, microarray and RNA Seq transcriptomics, and genetic analysis have revealed rhizobial (120, 121, 123), *Pseudomonas* (124, 125), *Streptomyces* (126), and other bacterial genes expressed on roots or rhizospheres. Similarly, bacteria may differentially express genes when in guts. Gut bacteria are exposed to bile salts that solubilize diet fat, have antimicrobial activities (127), and regulate bacterial gene expression. An efflux transporter of the multidrug resistance type (MDR) was induced in *Bifidobacterium* by bile (128). Different bile substances have been identified to control gene expression in bifidobacteria (129). Other bile-inducible genes have been found in *Lactobacillus plantarum* (130). Lastly, human gut bacteria transform bile salts (131). Gut bacteria can also modify dietary flavonoids (132) that have significant effects on animal physiology. Analogously, in roots, flavonoids produced by plants are signal molecules in bacteria (133) and are also transformed by bacteria *in vitro*, though this has not been shown *in vivo*. Plant phytoalexins are antimicrobials that are expelled from *Rhizobium etli*, *Bradyrhizobium japonicum*, and *Agrobacterium* by MDR efflux pumps that are inducible by root-exudated flavonoids (20, 119, 134).

Interestingly, gut and root microbiotas may follow the circadian cycles of their hosts. This was observed in nitrogen-fixing bacteria that fixed more during the daytime on rice roots (135). Epithelial cell proliferation, gastrointestinal motility, and other gut processes follow biological rhythms. In the gastrointestinal tract, there are large amounts of melatonin, which is a key hormone in the clock biological regulation (136). The Burmese python's microbiota is responsive to host cycles of feeding and fasting (137).

Host gene expression regulated by microbiotas. Outstandingly, gut and root bacteria modify gene expression in animal (138, 139) and plant (140) hosts, respectively. Gut gene expression is also modified by probiotics (113) that modify gut bacterial gene expression as well (141). Gut genes expressed in the presence of the gut bacterium *Bacteroides thetaiotaomicron* are involved in xenobiotic catabolism, in angiogenesis, in gut barrier epithelium maintenance, and in immunity development (139), with very complex host molecular responses (142).

Plants and humans can sense bacterially produced acylhomoserine lactones (AHLs), different volatiles, microbe-associated molecular patterns (MAMPS) (72, 143), and other bacterial molecules unknown at present. Root gene expression is differently modified by acylhomoserine lactones from pathogenic or symbiotic bacteria (144). In turn, plant products may act like quorum-sensing signals in bacteria (145). In recent years, specific regulatory roles of *N*-acylhomoserine lactones have become apparent, because plants responded with either a systemic resistance response or a hormonal regulated growth response to the presence of AHL-producing bacteria colonizing the root surface. Also in the animal/human systems, a specific perception of AHL compounds, produced by Gram-negative, mostly pathogenic bacteria, was found in many tissues, including the gut system, leading to immunomodulatory effects (146). In plants, root genes induced by rhizospheric bacteria are involved in oxidative and defense responses, in plant secondary metabolism, or in signaling (140). Plants may detect bacterial cyclopeptides through auxin sensing pathways (147). In a more specialized symbiosis, a cascade of signaling processes occurs inside root cells in the presence of rhizobia or Nod factors (148).

Control of microbiotas. A *Drosophila* mutant with increased levels of antimicrobial peptides showed deregulated balances of gut populations (149), with smaller numbers of *Commensalibacter intestini* (an acetic acid bacterium present in normal gut) bacteria (150) and increased numbers of *Gluconobacter morbifer* cells that caused gut cell apoptosis and early insect death (149). It is interesting to note that *C. intestini* antagonizes *G. morbifer*, which is a normal gut member, but with detrimental effects when present in large numbers; thus, *C. intestini* contributes to gut homeostasis and host fitness (151). Similarly, among root microbiotas, there are plant-pathogenic bacteria that normally would not affect the plants when kept in low numbers by other plant community strains or plant antimicrobials. Lipopolysaccharide *Rhizobium* mutants that were more sensitive to maize antimicrobial benzoxazinones had reduced rhizospheric colonization (152). Antimicrobial peptides constitute a line of defense in plants as effectors of innate immunity and regulate not only bacteria but also methanogenic archaea in guts (153). Gut immunity determines bacterial composition; reciprocally, bacteria modulate host immunity in guts (154, 155). Carbohydrate binding proteins (lectins) from guts and roots bind bacteria, form aggregates, and may have antibacterial effects (156, 157).

In addition to bacterium-host interactions, bacterium-bacterium interactions may determine community composition and its function (158). Those that occur in the mouth (159) may guide research in gut and root symbioses. In *Rhizobium*, mutants in quorum sensing are affected in rhizosphere colonization (160). Acylhomoserine lactones may be degraded by rhizospheric bacteria causing interference with quorum signals that regulate gene expression in other bacteria (161). This may have a role in protecting plants from pathogens but may also affect mutualistic interactions.

EVOLUTIONARY PATHWAYS

Lateral gene transfer in guts and roots. In roots, root nodules, and guts, lateral transfer of genetic material between different bacteria has been evidenced (2, 162, 163), seemingly promoted by close contacts in high-density populations. The presence of similar catabolic or antibiotic resistance genes in various gut bacterial genera has been explained as acquisitions by lateral gene transfers (91). It has been suggested that starch catabolism genes have been transferred from gut to bacteria (164).

There are many more phages than bacteria in the gut (13), and some may be involved in lateral gene transfer among gut bacteria (165). Lateral transfer of genetic material is mediated by plasmids or genomic island mobilization in rhizobia and other rhizospheric bacteria (54, 166), but phages may have a role as well.

Specialized symbiont evolution from root and gut bacteria. It has been suggested that gut bacteria gave rise to endosymbiotic bacteria in insects (167) based on similarities of gut bacteria and insect endosymbionts (168). Correspondingly, rhizospheric bacteria may have preceded nodule and endophytic bacteria in plants (169). Insect endosymbionts and nodule rhizobia are selected

symbionts that occupy intracellularly host-specialized structures and attain high numbers with a determined functional role. However, transmission modes of plant- and insect-specialized symbionts (reviewed in reference 46) and their genome sizes (rhizobial genome sizes reviewed in references 121 and 170) are different.

CONCLUSIONS

The comparison of plant and gut microbial ecologies may help to guide research toward the understanding of such complex symbioses. Literature on the subject is so extensive that only a few references were used to illustrate the commonalities of gut and root microbiotas. Interested readers are referred to recent literature (171–175). Plants use their "guts" (roots) outwards, and this simplifies their study in comparison to study of animal guts. Gut and root microbiotas significantly impact health, development, and fitness of their respective hosts.

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REFERENCES

1. **Badri DV, Weir TL, van der Lelie D, Vivanco JM.** 2009. Rhizosphere chemical dialogues: plant-microbe interactions. Curr. Opin. Biotechnol. **20**:642–650.
2. **Dillon RJ, Dillon VM.** 2004. The gut bacteria of insects: nonpathogenic interactions. Annu. Rev. Entomol. **49**:71–92.
3. **Kurokawa K, Itoh T, Kuwahara T, Oshima K, Toh H, Toyoda A, Takami H, Morita H, Sharma VK, Srivastava TP, Taylor TD, Noguchi H, Mori H, Ogura Y, Ehrlich DS, Itoh K, Takagi T, Sakaki Y, Hayashi T, Hattori M.** 2007. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. DNA Res. **14**:169–181.
4. **Marchesi JR.** 2010. Prokaryotic and eukaryotic diversity of the human gut. Adv. Appl. Microbiol. **72**:43–62.
5. **Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI.** 2007. The human microbiome project. Nature **449**:804–810.
6. **Donovan SE, Purdy KJ, Kane MD, Eggleton P.** 2004. Comparison of *Euryarchaea* strains in the guts and food-soil of the soil-feeding termite *Cubitermes fungifaber* across different soil types. Appl. Environ. Microbiol. **70**:3884–3892.
7. **Fricke WF, Seedorf H, Henne A, Krüer M, Liesegang H, Hedderich R, Gottschalk G, Thauer RK.** 2006. The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H_2 for methane formation and ATP synthesis. J. Bacteriol. **188**:642–658.
8. **Friedrich MW, Schmitt-Wagner D, Leuders T, Brune A.** 2001. Axial differences in community structure of *Crenarchaeota* and *Euryarchaeota* in the highly compartmentalized gut of the soil-feeding termite *Cubitermes orthognathus*. Appl. Environ. Microbiol. **67**:4880–4890.
9. **Hara K, Shinzato N, Seo M, Oshima T, Yamagishi A.** 2002. Phylogenetic analysis of symbiotic archaea living in the gut of xylophagous cockroaches. Microbes Environ. **17**:185–190.
10. **Horz HP, Conrads G.** 2010. The discussion goes on: what is the role of *Euryarchaeota* in humans? Archaea **2010**:967271.
11. **Jarrell KF, Walters AD, Bochiwal C, Borgia JM, Dickinson T, Chong JP.** 2011. Major players on the microbial stage: why archaea are important. Microbiology **157**:919–936.
12. **Simon HM, Dodsworth JA, Goodman RM.** 2000. Crenarchaeota colonize terrestrial plant roots. Environ. Microbiol. **2**:495–505.
13. **Minot S, Grunberg S, Wu GD, Lewis JD, Bushman FD.** 2012. Hypervariable loci in the human gut virome. Proc. Natl. Acad. Sci. U. S. A. **109**:3962–3966.
14. **Minot S, Sinha R, Chen J, Li H, Keilbaugh SA, Wu GD, Lewis JD, Bushman FD.** 2011. The human gut virome: inter-individual variation and dynamic response to diet. Genome Res. **21**:1616–1625.
15. **Reyes A, Haynes M, Hanson N, Angly FE, Heath AC, Rohwer F, Gordon JI.** 2010. Viruses in the faecal microbiota of monozygotic twins and their mothers. Nature **466**:334–338.
16. **Swanson MM, Fraser G, Daniell TJ, Torrance L, Gregory PJ, Taliansky M.** 2009. Viruses in soils: morphological diversity and abundance in the rhizosphere. Ann. Appl. Biol. **155**:51–60.
17. **Nam YD, Chang HW, Kim KH, Roh SW, Kim MS, Jung MJ, Lee SW, Kim JY, Yoon JH, Bae JW.** 2008. Bacterial, archaeal, and eukaryal diversity in the intestines of Korean people. J. Microbiol. **46**:491–501.
18. **Pandey PK, Siddharth J, Verma P, Bavdekar A, Patole MS, Shouche YS.** 2012. Molecular typing of fecal eukaryotic microbiota of human infants and their respective mothers. J. Biosci. **37**:221–226.
19. **Parfrey LW, Walters WA, Knight R.** 2011. Microbial eukaryotes in the human microbiome: ecology, evolution, and future directions. Front. Microbiol. **2**:153.
20. **Parniske M.** 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat. Rev. Microbiol. **6**:763–775.
21. **Bertaux J, Schmid M, Chemidlin Prevost-Boure N, Churin JL, Hartmann A, Garbaye J, Frey-Klett P.** 2003. *In situ* identification of intracellular bacteria related to *Paenibacillus* spp. in the mycelium of the ectomycorrhizal fungus *Laccaria* bicolor S238N. Appl. Environ. Microbiol. **69**:4243–4248.
22. **Bianciotto V, Lumini E, Bonfante P, Vandamme P.** 2003. '*Candidatus* Glomeribacter gigasporarum' gen. nov., sp. nov., an endosymbiont of arbuscular mycorrhizal fungi. Int. J. Syst. Evol. Microbiol. **53**:121–124.
23. **Scheublin TR, Sanders IR, Keel C, van der Meer JR.** 2010. Characterisation of microbial communities colonising the hyphal surfaces of arbuscular mycorrhizal fungi. ISME J. **4**:752–763.
24. **Stingl U, Radek R, Yang H, Brune A.** 2005. "Endomicrobia": cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. Appl. Environ. Microbiol. **71**:1473–1479.
25. **Biagi E, Candela M, Fairweather-Tait S, Franceschi C, Brigidi P.** 2012. Ageing of the human metaorganism: the microbial counterpart. Age **34**: 247–267.
26. **Mihajlovski A, Doré J, Levenez F, Alric M, Brugère JF.** 2010. Molecular evaluation of the human gut methanogenic archaeal microbiota reveals an age-associated increase of the diversity. Environ. Microbiol. Rep. **2**:272–280.
27. **Tiihonen K, Ouwehand AC, Rautonen N.** 2010. Human intestinal microbiota and healthy ageing. Ageing Res. Rev. **9**:107–116.
28. **Yatsunenko T, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights D, Knight R, Gordon JI.** 2012. Human gut microbiome viewed across age and geography. Nature **486**: 222–227.
29. **Muegge BD, Kuczynski J, Knights D, Clemente JC, González A, Fontana L, Henrissat B, Knight R, Gordon JI.** 2011. Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science **332**:970–974.
30. **Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI.** 2009. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci. Transl. Med. **1**:6ra14. doi: 10.1126/scitranslmed.3000322.
31. **Hooper LV, Midtvedt T, Gordon JI.** 2002. How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu. Rev. Nutr. **22**:283–307.
32. **Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, Schlegel ML, Tucker TA, Schrenzel MD, Knight R, Gordon JI.** 2008. Evolution of mammals and their gut microbes. Science **320**:1647–1651.
33. **Hong PY, Wheeler E, Cann IKO, Mackie RI.** 2011. Phylogenetic analysis of the fecal microbial community in herbivorous land and marine iguanas of the Galápagos Islands using 16S rRNA-based pyrosequencing. ISME J. **5**:1461–1470.
34. **Rosenblueth M, Martínez-Romero E.** 2006. Bacterial endophytes and their interactions with hosts. Mol. Plant Microbe Interact. **19**:827–837.
35. **Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, Tremblay J, Engelbrektson A, Kunin V, del Rio TG, Edgar RC, Eickhorst T, Ley RE, Hugenholtz P, Tringe SG, Dangl JL.** 2012. Defining the core *Arabidopsis thaliana* root microbiome. Nature **488**: 86–90.
36. **Hartmann A, Schmid M, van Tuinen D, Berg G.** 2009. Plant-driven selection of microbes. Plant Soil **321**:235–257.
37. **Chiarini L, Giovannelli V, Bevivino A, Dalmastri C, Tabacchioni S.** 2000. Different portions of the maize root system host *Burkholderia ce-*

pacia populations with different degrees of genetic polymorphism. Environ. Microbiol. **2**:111–118.
38. **Doornbos RF, van Loon LC, Bakker PAHM.** 2012. Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. Agron. Sustain. Dev. **32**:227–243.
39. **Rovira AD.** 1969. Plant root exudates. Bot. Rev. **35**:35–57.
40. **Chow J, Lee SM, Shen Y, Khosravi A, Mazmanian SK.** 2010. Host-bacterial symbiosis in health and disease. Adv. Immunol. **107**:243–274.
41. **Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moenne-Loccoz Y.** 2009. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil **321**:341–361.
42. **Bertin C, Yang X, Weston LA.** 2003. The role of root exudates and allelochemicals in the rhizosphere. Plant Soil **256**:67–83.
43. **Turroni F, Marchesi JR, Foroni E, Gueimonde M, Shanahan F, Margolles A, van Sinderen D, Ventura M.** 2009. Microbiomic analysis of the bifidobacterial population in the human distal gut free. ISME J. **3**:745–751.
44. **Marteau P, Pochart P, Doré J, Béra-Maillet C, Bernalier A, Corthier G.** 2001. Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl. Environ. Microbiol. **67**:4939–4942.
45. **Nava GM, Friedrichsen HJ, Stappenbeck TS.** 2011. Spatial organization of intestinal microbiota in the mouse ascending colon. ISME J. **5**:627–638.
46. **Kikuchi Y, Hosokawa T, Fukatsu T.** 2008. Diversity of bacterial symbiosis in stinkbugs, p 39–63. *In* Dijk TV (ed), Microbial Ecology Research Trends. Nova Science Publishers Inc., New York, NY.
47. **Brune A, Emerson D, Breznak JA.** 1995. The termite gut microflora as an oxygen sink—microelectrode determination of oxygen and pH gradients in guts of lower and higher termites. Appl. Environ. Microbiol. **61**:2681–2687.
48. **Timmers RA, Rothballer M, Strik DP, Engel M, Schulz S, Schloter M, Hartmann A, Hamelers B, Buisman C.** 2012. Microbial community structure elucidates performance of Glyceria maxima plant microbial fuel cell. Appl. Microbiol. Biotechnol. **94**:537–548.
49. **Asahara T, Shimizu K, Nomoto K, Hamabata T, Ozawa A, Takeda Y.** 2004. Probiotic bifidobacteria protect mice from lethal infection with Shiga toxin-producing *Escherichia coli* O157:H7. Infect. Immun. **72**: 2240–2247.
50. **Rodriguez H, Gonzalez T, Goire I, Bashan Y.** 2004. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. Naturwissenschaften **91**:552–555.
51. **Sharma S, Sharma S, Singh RK, Vaishampayan A.** 2008. Colonization behavior of bacterium *Burkholderia cepacia* inside the *Oryza sativa* roots visualized using green fluorescent protein reporter. World J. Microbiol. Biotechnol. **24**:1169–1175.
52. **Costello EK, Stagaman K, Dethlefsen L, Bohannan BJM, Relman DA.** 2012. The application of ecological theory toward an understanding of the human microbiome. Science **336**:1255–1262.
53. **Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA.** 2005. Diversity of the human intestinal microbial flora. Science **308**:1635–1638.
54. **López-López A, Rogel MA, Ormeño-Orrillo E, Martínez-Romero J, Martínez-Romero E.** 2010. *Phaseolus vulgaris* seed-borne endophytic community with novel bacterial species such as *Rhizobium endophyticum* sp. nov. Syst. Appl. Microbiol. **33**:322–327.
55. **Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D, Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Doré J, Weissenbach J, Ehrlich SD, Bork P.** 2011. Enterotypes of the human gut microbiome. Nature **473**:174–180.
56. **Friesen ML, Porter SS, Stark SC, von Wettberg EJ, Sachs JL, Martinez-Romero E.** 2011. Microbially mediated plant functional traits. Annu. Rev. Ecol. Evol. Syst. **42**:23–46.
57. **Rosenblueth M, Martínez-Romero E.** 2004. *Rhizobium etli* endophytic populations and their competitiveness for root maize colonization. Arch. Microbiol. **181**:337–344.
58. **Macfarlane S, Macfarlane GT.** 2003. Food and the large intestine, p 24–51. *In* Fuller R, Perdigon G (ed), Gut flora, nutrition, immunity and health, Blackwell Publishing, Oxford, United Kingdom.
59. **Kleerebezem M, Hols P, Bernard E, Rolain T, Zhou M, Siezen RJ, Bron PA.** 2010. The extracellular biology of the lactobacilli. FEMS Microbiol. Rev. **34**:199–230.
60. **Kikuchi Y, Hosokawa T, Fukatsu T.** 2011. An ancient but promiscuous host-symbiont association between *Burkholderia* gut symbionts and their heteropteran hosts. ISME J. **5**:446–460.
61. **Johnston-Monje D, Raizada MN.** 2011. Conservation and diversity of seed associated endophytes in *Zea* across boundaries of evolution, ethnography and ecology. PLoS One **6**:e20396. doi:10.1371/journal.pone.0020396.
62. **Pereira P, Ibánez F, Rosenblueth M, Etcheverry M, Martínez-Romero E.** 2011. Analysis of the bacterial diversity associated with the roots of maize (*Zea mays* L.) through culture-dependent and culture-independent methods. ISRN Ecol. **2011**:938546. doi:10.5402/2011/938546.
63. **Damiani C, Ricci I, Crotti E, Rossi P, Rizzi A, Scuppa P, Esposito F, Bandi C, Daffonchio D, Favia G.** 2008. Paternal transmission of symbiotic bacteria in malaria vectors. Curr. Biol. **18**:R1087–R1088.
64. **Jiménez E, Fernández L, Marín ML, Martín R, Odriozola JM, Nueno-Palop C, Narbad A, Olivares M, Xaus J, Rodríguez JM.** 2005. Isolation of commensal bacteria from umbilical cord blood of healthy neonates born by cesarean section. Curr. Microbiol. **51**:270–274.
65. **Jiménez E, Marín ML, Martín R, Odriozola JM, Olivares M, Xaus J, Fernández L, Rodríguez JM.** 2008. Is meconium from healthy newborns actually sterile? Res. Microbiol. **159**:187–193.
66. **Mshvildadze M, Neu J, Shuster J, Theriaque D, Li N, Mai V.** 2010. Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. J. Pediatr. **156**:20–25.
67. **Hunt KM, Foster JA, Forney LJ, Schütte UM, Beck DL, Abdo Z, Fox LK, Williams JE, McGuire MK, McGuire MA.** 2011. Characterization of the diversity and temporal stability of bacterial communities in human milk. PLoS One **6**:e21313. doi:10.1371/journal.pone.0021313.
68. **Martin R, Jiménez E, Heilig H, Fernandez L, Marín ML, Zoetendal EG, Rodriguez JM.** 2009. Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. Appl. Environ. Microbiol. **75**:965–969.
69. **Martín R, Langa S, Reviriego C, Jiménez E, Marín ML, Xaus J, Fernández L, Rodríguez JM.** 2003. Human milk is a source of lactic acid bacteria for the infant gut. J. Pediatr. **143**:754–758.
70. **Fernández A, Huang S, Seston S, Xing J, Hickey R, Criddle C, Tiedje J.** 1999. How stable is stable? Function versus community composition. Appl. Environ. Microbiol. **65**:3697–3704.
71. **Pérez-Chaia AP, Oliver G.** 2003. Intestinal microflora and metabolic activity, p 77–98. *In* Fuller R, Perdigon G (ed), Gut flora, nutrition, immunity and health. Blackwell Publishing, Oxford, United Kingdom.
72. **Ortíz-Castro R, Contreras-Cornejo HA, Macías-Rodríguez L, López-Bucio J.** 2009. The role of microbial signals in plant growth and development. Plant Signal Behav. **4**:701–712.
73. **Phillips DA, Martínez-Romero E, Yang GP, Joseph JM.** 2000. Release of nitrogen: a key trait in selecting bacterial endophytes for agronomically useful nitrogen fixation, p 205–217. *In* Ladha JK, Reddy PM (ed), The quest for nitrogen fixation in rice. IRRI, Manila, Philippines.
74. **Phillips DA, Joseph CM, Yang GP, Martinez-Romero E, Sanborn JR, Volpin H.** 1999. Identification of lumichrome as a sinorhizobium enhancer of alfalfa root respiration and shoot growth. Proc. Natl. Acad. Sci. U. S. A. **96**:12275–12280.
75. **Gibson GR, Cummings JH, Macfarlane GT.** 1988. Use of a three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. Appl. Environ. Microbiol. **54**:2750–2755.
76. **Ladha JK, Barraquio WL, Watanabe I.** 1983. Isolation and identification of nitrogen-fixing *Enterobacter cloacae* and *Klebsiella planticola* associated with rice plants. Can. J. Microbiol. **29**:1301–1308.
77. **Madhaiyan M, Poonguzhali S, Kwon SW, Sa TM.** 2009. *Methylophilus rhizosphaerae* sp. nov., a restricted facultative methylotroph isolated from rice rhizosphere soil. Int. J. Syst. Evol. Microbiol. **59**:2904–2908.
78. **St-Pierre B, Wright AD.** 27 April 2012, posting date. Diversity of gut methanogens in herbivorous animals. Animal. http://dx.doi.org/10.1017/S1751731112000912.
79. **Ormeño-Orrillo E, Hungria M, Martinez-Romero E.** Dinitrogen-fixing prokaryotes. *In* Rosenberg E, DeLong EF, Stackebrandt E, Lory S,

Thompson F (ed), The prokaryotes, vol 1. Symbiotic associations, biotechnology, applied microbiology, 4th ed, in press. Springer, New York, NY.
80. **Behar A, Yuval B, Jurkevitch E.** 2005. Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. Mol. Ecol. 14:2637–2643.
81. **Desai MS, Brunes A.** 2012. Bacteroidales ectosymbionts of gut flagellates shape the nitrogen-fixing community in dry-wood termites. ISME J. 6:1302–1313.
82. **Ohkuma M.** 2008. Symbioses of flagellates and prokaryotes in the gut of lower termites. Trends Microbiol. 16:345–352.
83. **Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA.** 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat. Rev. Microbiol. 6:121–131.
84. **Osawa R, Kuroiso K, Goto S, Shimizu A.** 2000. Isolation of tannin-degrading lactobacilli from humans and fermented foods. Appl. Environ. Microbiol. 66:3093–3097.
85. **Gérard P, Lepercq P, Leclerc M, Gavini F, Raibaud P, Juste C.** 2007. *Bacteroides* sp. strain D8, the first cholesterol-reducing bacterium isolated from human feces. Appl. Environ. Microbiol. 73:5742–5749.
86. **Derrien M, Collado MC, Ben-Amor K, Salminen S, de Vos WM.** 2008. The mucin degrader *Akkermansia muciniphila* is an abundant resident of the human intestinal tract. Appl. Environ. Microbiol. 74:1646–1648.
87. **Calvaruso C, Turpault MP, Frey-Klett P.** 2006. Root-associated bacteria contribute to mineral weathering and to mineral nutrition in trees: a budgeting analysis. Appl. Environ. Microbiol. 72:1258–1266.
88. **Puente ME, Bashan Y, Li CY, Lebsky VK.** 2004. Microbial populations and activities in the rhizoplane of rock-weathering desert plants. I. Root colonization and weathering of igneous rocks. Plant Biol. 6:629–642.
89. **Soedarjo M, Hemscheidt TK, Borthakur D.** 1994. Mimosine, a toxin present in leguminous trees (*Leucaena* spp.), induces a mimosine-degrading enzyme activity in some *Rhizobium* strains. Appl. Environ. Microbiol. 60:4268–4272.
90. **Allison MJ, Hammond AC, Jones RJ.** 1990. Detection of ruminal bacteria that degrade toxic dihydroxypyridine compounds produced from mimosine. Appl. Environ. Microbiol. 56:590–594.
91. **Thomas F, Barbeyron T, Tonon T, Génicot S, Czjzek M, Michel G.** 2012. Characterization of the first alginolytic operons in a marine bacterium: from their emergence in marine Flavobacteria to their independent transfers to marine Proteobacteria and human gut Bacteroides. Environ. Microbiol. 14:2379–2394.
92. **Kuiper I, Lagendijk EL, Bloemberg GV, Lugtenberg BJ.** 2004. Rhizoremediation: a beneficial plant-microbe interaction. Mol. Plant Microbe Interact. 17:6–15.
93. **Lugtenberg BJ, Kravchenko LV, Simons M.** 1999. Tomato seed and root exudate sugars: composition, utilization by *Pseudomonas* biocontrol strains and role in rhizosphere colonization. Environ. Microbiol. 1:439–446.
94. **Haiser HJ, Turnbaugh PJ.** 2012. Is it time for a metagenomic basis of therapeutics? Science 336:1253–1255.
95. **Mikov M.** 1994. The metabolism of drugs by the gut flora. Eur. J. Drug Metab. Pharmacokinet. 19:201–207.
96. **Sousa T, Paterson R, Moore V, Carlsson A, Abrahamsson B, Basit AW.** 2008. The gastrointestinal microbiota as a site for the biotransformation of drugs. Int. J. Pharm. 363:1–25.
97. **Pham TT, Tu Y, Sylvestre M.** 2012. Remarkable ability of *Pandoraea pnomenusa* B356 biphenyl dioxygenase to metabolize simple flavonoids. Appl. Environ. Microbiol. 78:3560–3570.
98. **Rodionov DA, Vitreschak AG, Mironov AA, Gelfand MS.** 2003. Comparative genomics of the vitamin B12 metabolism and regulation in prokaryotes. J. Biol. Chem. 278:41148–41159.
99. **Albert MJ, Mathan VI, Baker SJ.** 1980. Vitamin B12 synthesis by human small intestinal bacteria. Nature 283:781–782.
100. **Campbell GR, Taga ME, Mistry K, Lloret J, Anderson PJ, Roth JR, Walker GC.** 2006. *Sinorhizobium meliloti bluB* is necessary for production of 5,6-dimethylbenzimidazole, the lower ligand of B12. Proc. Natl. Acad. Sci. U. S. A. 103:4634–4639.
101. **Morita H, Toh H, Fukuda S, Horikawa H, Oshima K, Suzuki T, Murakami M, Hisamatsu S, Kato Y, Takizawa T, Fukuoka H, Yshimura T, Itoh K, O'Sullivan D, McKay L, Ohno H, Kikuchi J, Masaoka T, Hattori M.** 2008. Comparative genome analysis of *Lactobacillus reuteri* and *Lactobacillus fermentum* reveal a genomic island for reuterin and cobalamin production. DNA Res. 15:151–161.
102. **Ramotar K, Conly JM, Chubb H, Louie TJ.** 1984. Production of menaquinones by intestinal anaerobes. J. Infect. Dis. 150:213–218.
103. **Shin SC, Kim SH, You H, Kim B, Kim AC, Lee KA, Yoon JH, Ryu JH, Lee WJ.** 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334:670–674.
104. **Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhasset P, Macri J, McCoy K, Verdu EF, Collins SM.** 2011. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141:599–609.
105. **Díaz Heijtz RD, Wang S, Anuar F, Qiuan Y, Björkholm B, Samuelsson A, Hibberd ML, Frossberg H, Pettersson S.** 2011. Normal gut microbiota modulates brain development and behavior. Proc. Natl. Acad. Sci. U. S. A. 108:3047–3052.
106. **Kane M, Case LK, Kopaskie K, Kozlova A, MacDearmid C, Chervonsky AV, Golovkina TV.** 2011. Successful transmission of a retrovirus depends on the commensal microbiota. Science 334:245–249.
107. **Jung SC, Martínez-Medina A, López-Raez JA, Pozo MJ.** 2012. Mycorrhiza-induced resistance and priming of plant defenses. J. Chem. Ecol. 38:651 664.
108. **Saxelin M, Tynkkynen S, Mattila-Sandholm T, de Vos WM.** 2005. Probiotic and other functional microbes: from markets to mechanisms. Curr. Opin. Biotechnol. 16:204–211.
109. **Puente ME, Li CY, Bashan Y.** 2009. Rock-degrading endophytic bacteria in cacti. Environ. Exp. Bot. 66:389–401.
110. **Gibson GR, Rastall RA, Fuller R.** 2003. The health benefits of probiotics and prebiotics, p 52–76. *In* Fuller R, Perdigon G (ed), Gut flora, nutrition, immunity and health. Blackwell Publishing, Oxford, United Kingdom.
111. **Rosenblueth M, Hynes MF, Martínez-Romero E.** 1998. *Rhizobium tropici teu* genes involved in specific uptake of *Phaseolus vulgaris* bean-exudate compounds. Mol. Gen. Genet. 258:587–598.
112. **Tepfer D, Goldmann A, Pamboukdjian N, Maille M, Lepingle A, Chevalier D, Dénarié J, Rosenberg C.** 1988. A plasmid of *Rhizobium meliloti* 41 encodes catabolism of two compounds from root exudate of *Calystegium sepium*. J. Bacteriol. 170:1153–1161.
113. **van Baarlen P, Troost F, van der Meer C, Hooiveld G, Boekschoten M, Brummer RJM, Kleerebezem M.** 2011. Human mucosal in vivo transcriptome responses to three lactobacilli indicate how probiotics may modulate human cellular pathways. Proc. Natl. Acad. Sci. U. S. A. 108(Suppl 1):4562–4569.
114. **Floch MH, Walker WA.** 2008. Advances in clinical use of probiotics. J. Clin. Gastroenterol. 42:S45.
115. **Zimmer W, Stephan MP, Bothe H.** 1984. Denitrification by *Azospirillum brasilense* Sp 7. Arch. Microbiol. 138:206–211.
116. **Delgado S, O'Sullivan E, Fitzgerald G, Mayo B.** 2008. *In vitro* evaluation of the probiotic properties of human intestinal *Bifidobacterium* species and selection of new probiotic candidates. J. Appl. Microbiol. 104: 1119–1127.
117. **Peix A, Velázquez E, Silva LR, Mateos PF.** 2010. Key molecules involved in beneficial infection process in rhizobia-legume symbiosis, p 55–80. *In* Khan MH, Zaidi A, Musarrat J (ed), Microbes for legume improvement. Springer, Vienna, Austria.
118. **Downie JA.** 2010. The roles of extracellular proteins, polysaccharides and signals in the interactions of rhizobia with legume roots. FEMS Microbiol. Rev. 34:150–170.
119. **González-Pasayo R, Martínez-Romero E.** 2000. Multiresistance genes of *Rhizobium etli* CFN42. Mol. Plant Microbe Interact. 13:572–577.
120. **Ramachandran VK, East AK, Karunakaran R, Downie JA, Poole PS.** 2011. Adaptation of *Rhizobium leguminosarum* to pea, alfalfa and sugar beet rhizospheres investigated by comparative transcriptomics. Genome Biol. 12:R106.
121. **López-Guerrero MG, Ormeño-Orrillo E, Acosta JL, Mendoza-Vargas A, Rogel MA, Ramírez MA, Rosenblueth M, Martínez-Romero J, Martínez-Romero E.** 2012. Rhizobial extrachromosomal replicon variability, stability and expression in natural niches. Plasmid 68:149–158.
122. **Ramos-González MI, Campos MJ, Ramos JL.** 2005. Analysis of *Pseudomonas putida* KT2440 gene expression in the maize rhizosphere: *in vitro* expression technology capture and identification of root-activated promoters. J. Bacteriol. 187:4033–4041.
123. **Karunakaran R, Ramachandran VK, Seaman JC, East AK, Mouhsine B.** 2009. Transcriptomic analysis of rhizobium leguminosarum biovar

viciae in symbiosis with host plants *Pisum sativum* and *Vicia cracca*. J. Bacteriol. **191**:4002–4014.
124. **Espinosa-Urgel M, Ramos JL.** 2001. Expression of a *Pseudomonas putida* aminotransferase involved in lysine catabolism is induced in the rhizosphere. Appl. Environ. Microbiol. **67**:5219–5224.
125. **Kim YC, Miller CD, Anderson AJ.** 2000. Superoxide dismutase activity in *Pseudomonas putida* affects utilization of sugars and growth on root surfaces. Appl. Environ. Microbiol. **66**:1460–1467.
126. **Langlois P, Bourassa S, Poirier GG, Beaulieu C.** 2003. Identification of *Streptomyces coelicolor* proteins that are differentially expressed in the presence of plant material. Appl. Environ. Microbiol. **69**:1884–1889.
127. **Begley M, Gahan CGM, Hill C.** 2005. The interaction between bacteria and bile. FEMS Microbiol. Rev. **29**:625–651.
128. **Gueimonde M, Garrigues C, van Sinderen D, de los Reyes-Gavilán CG, Margolles A.** 2009. Bile-inducible efflux transporter from *Bifidobacterium longum* NCC2705, conferring bile resistance. Appl. Environ. Microbiol. **75**:3153–3160.
129. **Ruiz L, Alvarez-Martín P, Mayo B, de los Reyes-Gavilán CG, Gueimonde M, Margolles A.** 2012. Controlled gene expression in *Bifidobacteria* by use of a bile-responsive element. Appl. Environ. Microbiol. **78**: 581–585.
130. **Bron PA, Marco M, Hoffer SM, Van Mullekom E, de Vos MW, Kleerebezem M.** 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. J. Bacteriol. **186**:7829–7835.
131. **Ridlon JM, Kang DJ, Hylemon PB.** 2006. Bile salt biotransformations by human intestinal bacteria. J. Lipid Res. **47**:241–259.
132. **Blaut M, Schoefer L, Braune A.** 2003. Transformation of flavonoids by intestinal microorganisms. Int. J. Vitam. Nutr. Res. **73**:79–87.
133. **Cooper J.** 2004. Multiple responses of rhizobia to flavonoids during legume root infection. Adv. Bot. Res. **41**:1–62.
134. **Palumbo JD, Kado CI, Phillips DA.** 1998. An isoflavonoid-inducible efflux pump in *Agrobacterium tumefaciens* is involved in competitive colonization of roots. J. Bacteriol. **180**:3107–3113.
135. **Sims GK, Dunigan EP.** 1984. Diurnal and seasonal variations in nitrogenase activity (C_2H_2 reduction) of rice roots. Soil Biol. Biochem. **16**:15–18.
136. **Hoogerwerf WA.** 2006. Biologic clocks and the gut. Curr. Gastroenterol. Rep. **8**:353–359.
137. **Costello EK, Gordon JI, Secor SM, Knight R.** 2010. Postprandial remodeling of the gut microbiota in Burmese pythons. ISME J. **4**:1375–1385.
138. **Comelli EM, Simmering R, Faure M, Donnicola D, Mansourian R, Rochat F, Corthesy-Theulaz I, Cherbut C.** 2008. Multifaceted transcriptional regulation of the murine intestinal mucus layer by endogenous microbiota. Genomics **91**:70–77.
139. **Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science **291**:881–884.
140. **Rudrappa T, Czymmek KJ, Paré PW, Bais HP.** 2008. Root-secreted malic acid recruits beneficial soil bacteria. Plant Physiol. **148**:1547–1556.
141. **McNulty NP, Yatsunenko T, Hsiao A, Faith JJ, Muegge BD, Goodman AL, Henrissat B, Oozeer R, Cools-Portier S, Gobert G, Chervaux C, Knights D, Lozupone CA, Knight R, Duncan AE, Bain JR, Muehlbauer MJ, Newgard CB, Heath AC, Gordon JI.** 2011. The impact of a consortium of fermented milk strains on the gut microbiome of gnotobiotic mice and monozygotic twins. Sci. Transl. Med. **3**:106.
142. **Macpherson AJ, Harris NL.** 2004. Interactions between commensal intestinal bacteria and the immune system. Nat. Rev. Immunol. **4**:478–485.
143. **Farag MA, Ryu CM, Sumner LW, Paré PW.** 2006. GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry **67**:2262–2268.
144. **Mathesius M, Mulders S, Gao M, Teplitski M, Caetano-Anollés G, Rolfe BG, Bauer WD.** 2003. Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. Proc. Natl. Acad. Sci. U. S. A. **100**:1444–1449.
145. **Gao M, Teplitski M, Robinson JB, Bauer WD.** 2003. Production of substances by *Medicago truncatula* that affect bacterial quorum sensing. Mol. Plant Microbe Interact. **16**:827–834.
146. **Teplitski M, Mathesius U, Rumbaugh KP.** 2011. Perception and degradation of *N*-acyl homoserine lactone quorum sensing signals by mammalian and plant cells. Chem. Rev. **111**:100–116.
147. **Ortiz-Castro R, Díaz-Pérez C, Martínez-Trujillo M, del Río RE, Campos-García J, López-Bucio J.** 2011. Transkingdom signaling based on bacterial cyclodipeptides with auxin activity in plants. Proc. Natl. Acad. Sci. U. S. A. **108**:7253–7258.
148. **Oldroyd GE, Murray JD, Poole PS, Downie JA.** 2011. The rules of engagement in the legume-rhizobial symbiosis. Annu. Rev. Genet. **45**: 119–144.
149. **Ryu JH, Kim SH, Lee HY, Bai JY, Nam YD, Bae JW, Lee DG, Shin SC, Ha EM, Lee WJ.** 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. Science **319**: 777–782.
150. **Kim EK, Kim SH, Nam HJ, Choi MK, Lee KA, Choi SH, Seo YY, You H, Kim B, Lee WJ.** 2012b. Draft genome sequence of *Commensalibacter intestini* A911T, a symbiotic bacterium isolated from *Drosophila melanogaster* intestine. J. Bacteriol. **194**:1246.
151. **Roh SW, Nam YD, Chang HW, Kim KH, Kim MS, Ryu JH, Kim SH, Lee WJ, Bae JW.** 2008. Phylogenetic characterization of two novel commensal bacteria involved with innate immune homeostasis in *Drosophila melanogaster*. Appl. Environ. Microbiol. **74**:6171–6177.
152. **Ormeño-Orrillo E, Rosenblueth M, Luyten E, Vanderleyden J, Martínez-Romero E.** 2008. Mutations in lipopolysaccharide biosynthetic genes impair maize rhizosphere and root colonization of *Rhizobium tropici* CIAT899. Environ. Microbiol. **10**:1271–1284.
153. **Bang C, Schilhabel A, Weidenbach K, Kopp A, Goldmann T, Gutsmann T, Schmitz RA.** 2012. Effects of antimicrobial peptides on methanogenic archaea. Antimicrob. Agents Chemother. **56**:4123–4130.
154. **Lee WJ.** 2009. Bacterial-modulated host immunity and stem cell activation for gut homeostasis. Genes Dev. **23**:2260–2265.
155. **Round JL, Mazmanian SK.** 2009. The gut microbiota shapes intestinal immune responses during health and disease. Nat. Rev. Immunol. **9**:313–323.
156. **Peumans WJ, Van Damme EJ.** 1995. Lectins as plant defense proteins. Plant Physiol. **109**:347–352.
157. **Vaishnava S, Yamamoto M, Severson KM, Ruhn KA, Yu X, Koren O, Ley R, Wakeland EK, Hooper LV.** 2011. The antibacterial lectin RegIIIγ promotes the spatial segregation of microbiota and host in the intestine. Science **334**:255–258.
158. **Gibson GR, Wang X.** 1994. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J. Appl. Microbiol. **77**:412–420.
159. **Kreth J, Merritt J, Qi F.** 2009. Bacterial and host interactions of oral streptococci. DNA Cell Biol. **28**:397–403.
160. **Edwards A, Frederix M, Wisniewski-Dyé F, Jones J, Zorreguieta A, Downie JA.** 2009. The cin and rai quorum-sensing regulatory systems in *Rhizobium leguminosarum* are coordinated by ExpR and CinS, a small regulatory protein coexpressed with CinI. J. Bacteriol. **191**: 3059–3067.
161. **Jafra S, Przysowa J, Czajkowski R, Michta A, Garbeva P, Van der Wolf JM.** 2006. Detection and characterization of bacteria from the potato rhizosphere degrading *N*-acyl-homoserine lactone. Can. J. Microbiol. **52**:1006–1015.
162. **Kroer N, Barkay T, Sorensen S, Weber D.** 1998. Effect of root exudates and bacterial metabolic activity on conjugal gene transfer in the rhizosphere of a marsh plant. FEMS Microbiol. Ecol. **25**:375–384.
163. **Shoemaker NB, Vlamakis H, Hayes K, Salyers AA.** 2001. Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. Appl. Environ. Microbiol. **67**:561–568.
164. **Arias MC, Danchin EGJ, Coutinho PM, Henrissat B, Ball S.** 2012. Eukaryote to gut bacteria transfer of a glycoside hydrolase gene essential for starch breakdown in plants. Mob. Genet. Elements **2**:81–87.
165. **Stern A, Mick E, Tirosh I, Sagy O, Sorek R.** 2012. CRISPR targeting reveals a reservoir of common phages associated with the human gut microbiome. Genome Res. **22**:1985–1994.
166. **Sullivan JT, Ronson CW.** 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc. Natl. Acad. Sci. U. S. A. **95**:5145–5149.
167. **Husník F, Chrudimský T, Hypša V.** 2011. Multiple origins of endosymbiosis within the Enterobacteriaceae (γ-Proteobacteria): convergence of complex phylogenetic approaches. BMC Biol. **9**:87.
168. **Fukatsu T, Hosokawa T.** 2002. Capsule-transmitted gut symbiotic bac-

terium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. Appl. Environ. Microbiol. **68**:389–396.
169. **López-López A, Rosenblueth R, Martínez J, Martínez-Romero E.** 2010. Rhizobial symbioses in tropical legumes and non-legumes. Soil Biol. **21**: 163–184.
170. **McCutcheon JP, Moran NA.** 2012. Extreme genome reduction in symbiotic bacteria. Nat. Rev. Microbiol. **10**:13–26.
171. **de Bruijn FJ.** 2013. Molecular microbial ecology of the rhizosphere, vol I and II. Wiley-Blackwell, Hoboken, New Jersey.
172. **Dessaux Y, Hinsinger P, Lemanceau P.** 2007. Rhizosphere: achievements and challenges. Springer Science Press, Berlin, Germany.
173. **Fuller R, Perdigon G.** 2003. Gut flora, nutrition, immunity and health. Blackwell Publishing, Oxford, United Kingdom.
174. **Pinto R, Varanini Z, Nannipieri P.** 2007. The rhizosphere: biochemistry and organic substances at the soil-plant interface. Taylor and Francis Group/CRC Press, Boca Raton, FL.
175. **Sadowsky MJ, Whitman RL.** 2011. The fecal bacteria. ASM Press, Washington, DC.

REFERENCIAS

[1] Baumann, P. (2005). Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annual Review of Microbiology*, *59*, 155–89.

[2] Baumann, P., Moran, N. A., Baumann, L. (2013). Bacteriocyte-associated endosymbionts of insects In Rosenberg et al. (Ed.), *The Prokaryotes-- Prokaryotic biology and symbiotic associations*. (pp. 545-577) Springer-Verlag.

[3] Bianconi, E., Piovesan, A., Facchin, F., Beraudi, A., Casadei, R., Frabetti, F., Vitale, L., Pelleri, M.C., Tassani, S., Piva, F., Perez-Amodio, S., Strippoli, P., Canaider, S. (2013). An estimation of the number of cells in the human body. *Annals of Human Biology*, *40*(6), 463–71.

[4] Blanc, G., Ogata, H., Robert, C., Audic, S., Suhre, K., Vestris, G., Claverie, J.M., Raoult, D. (2007). Reductive genome evolution from the mother of Rickettsia. *PLoS Genetics*, *3*(1), 0103–0114.

[5] Brownlie, J. C., Cass, B. N., Riegler, M., Witsenburg, J. J., Iturbe-Ormaetxe, I., McGraw, E.A., O'Neill, S. L. (2009). Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. *PLoS Pathogens*, *5*(4), e1000368.

[6] Brusca, R. C., Brusca, G. J. (2003). *Invertebrates* (2nd ed.). Sinauer Associates, Incorporated.

[7] Buchner, P. (1965). *Endosymbiosis of animals with plant microorganisms*. (907 pp.) Interscience Publishers.

[8] Burke, G., Fiehn, O., Moran, N. (2010). Effects of facultative symbionts and heat stress on the metabolome of pea aphids. *The ISME Journal*, *4*(2), 242–52.

[9] Chávez-Moreno, C. K., Tecante, A., Casas, A. (2009). The *Opuntia* (Cactaceae) and *Dactylopius* (Hemiptera: Dactylopiidae) in Mexico: a historical perspective of use, interaction and distribution. *Biodiversity and Conservation*, *18*(13), 3337–3355.

[10] Chrostek, E., Marialva, M. S. P., Esteves, S. S., Weinert, L. A, Martinez, J., Jiggins, F. M., Teixeira, L. (2013). *Wolbachia* Variants Induce Differential Protection to Viruses in *Drosophila melanogaster*: A Phenotypic and Phylogenomic Analysis. *PLoS Genetics*, *9*(12), e1003896.

[11] Comandatore, F., Sassera, D., Montagna, M., Kumar, S., Koutsovoulos, G., Thomas, G., Repton, C., Babayan, S.A., Gray, N., Cordaux, R., Darby, A., Makepeace, B., Blaxter, M. (2013) Phylogenomics and analysis of shared genes suggest a single transition to mutualism in *Wolbachia* of nematodes. Genome Biol. Evol. 5, 1668–1674.

[12] Dale, C., Maudlin, I. (1999) *Sodalis* gen. nov. and *Sodalis glossinidius* sp. Nov., a microaerophilic secondary endosymbiont of the tsetse fly *Glossina morsitans morsitans*. Inter J Syst Bacteriol. 49, 267-275.

[13] Dapson, R. (2005). A method for determining identity and relative purity of carmine, carminic acid and aminocarminic acid. *Biotechnic & Histochemistry : Official Publication of the Biological Stain Commission*, *80*(5-6), 201–5.

[14] Darby, A. C., Armstrong, S. D., Bah, G. S., Kaur, G., Hughes, M. A., Kay, S. M., Koldkjaer, P., Rainbow, L., Radford, A. D., Blaxter, M. L., Tanya, V. N., Trees, A. J., Cordaux, R., Wastling, J. M., Makepeace, B. L. (2012). Analysis of gene expression from the *Wolbachia* genome of a filarial nematode supports both metabolic and defensive roles within the symbiosis. *Genome Research*, *22*, 2467–2477.

[15] Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M. E., Boulétreau, M. (2001). Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proceedings of the National Academy of Sciences*, *98*(11), 6247–6252.

[16] Derrien, M., van Hylckama Vlieg, J. E. (2015) Fate, activity and impact of ingested bacteria within the human gut microbiota. Trends Microbiol. 23(6), 354-366.

[17] Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Björkholm, B., Samuelsson, A., Hibberd, M. L., Forssberg, H., Pettersson, S. (2011). Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(7), 3047–52.

[18] Dodson, B. L., Hughes, G. L., Paul, O., Matacchiero, A. C., Kramer, L. D., Rasgon, J. L. 2014. Wolbachia enhances West Nile Virus (WNV) infection in the mosquito Culex tarsalis. PLOS Neg Trop Dis. 8(7): e2965.

[19] Douglas, A. E. (1998). Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. *Annual Review of Entomology*, *43*, 17–37. doi:10.1146/annurev.ento.43.1.17

[20] Douglas, A. E. (2012). “Can’t Live without You:” Essential Animal-Bacterial Relationships. *Microbe*, *7*(6), 273–277.

[21] Douglas-Escobar, M., Elliott, E., Neu, J. (2013). Effect of intestinal microbial ecology on the developing brain. *JAMA Pediatrics*, *167*(4), 374–9.

[22] Dubilier, N., Bergin, C., Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews. Microbiology*, *6*(10), 725–740.

[23] Duplouy, A., Iturbe-ormaetxe, I., Beatson, S. A., Szubert, J. M., Brownlie, J. C., Mcmeniman, C. J., McGraw, E. A., Hurst, G. D. D., Charlat, S., O’Neill, S. L., Woolfit, M. (2013). Draft genome sequence of the male-killing *Wolbachia* strain wBol1 reveals recent horizontal gene transfers from diverse sources. *BMC Genomics*.

[24] Eisner, T., Nowicki, S., Goetz, M., & Meinwald, J. (1980). Red Cochineal Dye (Carminic Acid): Its Role in Nature. *Science*, *208*(May), 1039–1042.

[25] Flores Coronado, V. (2011). Efecto de la fertilización de nopal (*Opuntia ficus-indica*) sobre la productividad y calidad de grana cochinilla *(Dactylopius coccus Costa)*.

[26] Fraune, S., Bosch, T. C. G. (2010). Why bacteria matter in animal development and evolution. *BioEssays*, *32*(7), 571–580. doi:10.1002/bies.200900192

[27] Furness, J. B., Callaghan, B. P., Rivera, L. R., Cho, H. (2014). *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease* (Vol. 817).

[28] Gil, R., Latorre, A., Moya, A. (2004). Bacterial endosymbionts of insects: insights from comparative genomics. *Environmental Microbiology*, *6*(11), 1109–22.

[29] Ginestra, G., Parker, M. L., Bennett, R. N., Robertson, J., Mandalari, G., Narbad, A., Lo Curto, R. B., Bisignano, G., Faulds, C. B., Waldron, K. W. (2009). Anatomical, chemical, and biochemical characterization of cladodes from prickly pear [*Opuntia ficus-indica* (L.) Mill.]. *Journal of Agricultural and Food Chemistry*, *57*(21), 10323–30.

[30] Hentschel, U., Piel, J., Degnan, S. M., Taylor, M. W. (2012). Genomic insights into the marine sponge microbiome. *Nature Reviews Microbiology*, *10*(9), 641–654.

[31] Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., Werren, J. H. (2008). How many species are infected with *Wolbachia*? A statistical analysis of current data. *FEMS Microbiology Letters*, *281*, 215–220.

[32] Huigens, M. E., Luck, R. F., Klaassen, R. H., Maas, M. F., Timmermans, M. J., Stouthamer, R. (2000). Infectious parthenogenesis. *Nature*, *405*(6783), 178–179.

[33] Ijichi, N., Kondo, N., Matsumoto, R., Shimada M., Ishikawa, H., Fukatsu, T. (2002) Internal spatiotemporal population dynamics of infection with three Wolbachia strains in the Adzuki bean beetle, Callosobruchus chinensis (Coleoptera: Bruchidae). Appl. Environ. Microbiol. 68(8), 4074-4080.

[34] Iturbe-ormaetxe, I., Burke, G. R., Riegler, M., O'Neill, S. L. (2005). Distribution, Expression, and Motif Variability of Ankyrin Domain Genes in *Wolbachia* pipientis. *Journal of Bacteriology*, *187*(15), 5136–5145.

[35] Iturbe-ormaetxe, I., Walker, T., O'Neill, S., 2011. *Wolbachia* and the biological control of mosquito-borne disease. EMBO Reports. 12(6): 508-518.

[36] Kikuchi, Y., Fukatsu, T. (2013). Live imaging of symbiosis: spatiotemporal infection dynamics of a GFP-labelled Burkholderia symbiont in the bean bug Riptortus pedestris.*Molecular Ecology*, *23*(6), 1445–56.

[37] Kikuchi, Y., Hosokawa, T., Nikoh, N., Meng, X.-Y., Kamagata, Y., Fukatsu, T. (2009). Host-symbiont co-speciation and reductive genome evolution in gut symbiotic bacteria of acanthosomatid stinkbugs. *BMC Biology*, *7*, 2.

[38] Koga, R., Meng, X.-Y., Tsuchida, T., Fukatsu, T. (2012). Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte-embryo interface. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(20), E1230–7.

[39] Koutsovoulos, G., Makepeace, B., Tanya, V.N., Blaxter, M. (2014) Palaeosymbiosis revealed by genomic fossils of *Wolbachia* in a Strongyloidean nematode. PLoS Genet. 10, e1004397.

[40] Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C., Salzberg, S. L. (2004). Versatile and open software for comparing large genomes.

[41] Landmann, F., Foster, J. M., Michalski, M. L., Slatko, B. E., Sullivan, W. (2014). Co-evolution between an Endosymbiont and Its Nematode Host: *Wolbachia* Asymmetric Posterior Localization and AP Polarity Establishment. *PLoS Neglected Tropical Diseases*, *8*(8), e3096.

[42] Ley, R. E., Peterson, D. A., Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, *124*(4), 837–48.

[43] Mathenge, C. W., Holford, P., Hoffmann, J. H., Spooner-Hart, R., Beattie, G. C., Zimmermann, H. G. (2009). The biology of *Dactylopius tomentosus* (Hemiptera: Dactylopiidae). *Bulletin of Entomological Research*, *99*(6), 551–9.

[44] McCutcheon, J. P., McDonald, B. R., Moran, N. (2009a). Convergent evolution of metabolic roles in bacterial co-symbionts of insects. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(36), 15394–9.

[45] McCutcheon, J. P., McDonald, B. R., Moran, N. (2009b). Origin of an alternative genetic code in the extremely small and GC-rich genome of a bacterial symbiont. *PLoS Genetics*, *5*(7), e1000565.

[46] McCutcheon, J. P., von Dohlen, C. D. (2011). An interdependent metabolic patchwork in the nested symbiosis of mealybugs. *Current Biology : CB*, *21*(16), 1366–72.

[47] Mcfall-Ngai, M. (2008). Are biologists in "future shock"? Symbiosis integrates biology across domains. *Nature Reviews. Microbiology*, *6*, 789–792.

[48] McMeniman, C. J., Lane, A. M., Fong, A. W. C., Voronin, D. A., Iturbe-Ormaetxe, I., Yamada, R., McGraw, E. A., O'Nell, S. L. (2008) Host adaptation of a *Wolbachia* strain after long-term serial passage in mosquito cell lines. Appl. Environ. Mcrobiol. 74(22), 6963-6969.

[49] Moran, N. (2006). Symbiosis. *Current Biology : CB*, *16*(20), R866–71.

[50] Moran, N. (2007). Symbiosis as an adaptive process and source of phenotypic complexity. *Proceedings of the National Academy of Sciences of the United States of America*, *104 Suppl*, 8627–33.

[51] Moran, N., McCutcheon, J. P., Nakabachi, A. (2008). Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics*, *42*, 165–90.

[52] Mouton, L., Dedeine, F., Henri, H., Boulétreau, M., Profizi, N., Vavre, F. (2004) Virulence, multiple infections and regulation of symbiotic population in the *Wolbachia-Asobara tabida* symbiosis. Genetics. 168, 181-189.

[53] Moya, A., Peretó, J., Gil, R., Latorre, A. (2008). Learning how to live together: genomic insights into prokaryote-animal symbioses. *Nature Reviews. Genetics*, *9*(3), 218–29.

[54] Nakabachi, A., Ishikawa, H. (1999). Provision of riboflavin to the host aphid, Acyrthosiphon pisum, by endosymbiotic bacteria, Buchnera. *Journal of Insect Physiology*, *45*(1), 1–6.

[55] Nikoh, N., Hosokawa, T., Moriyama, M., Oshima, K., Hattori, M., Fukatsu, T. (2014). Evolutionary origin of insect-*Wolbachia* nutritional mutualism. *Proceedings of the National Academy of Sciences*, (24), 4–6.

[56] Nyholm, S. V., McFall-Ngai, M. J. (2004). The winnowing: establishing the squid-vibrio symbiosis. *Nature Reviews. Microbiology*, *2*(8), 632–642.

[57] Pankewitz, F., Zöllmer, A., Hilker, M., & Gräser, Y. (2007). Presence of *Wolbachia* in insect eggs containing antimicrobially active anthraquinones. *Microbial Ecology*, *54*(4), 713–21.

[58] Plé, C., .Breton, J., Foligné, B. (2015) Maintaining gut ecosystems for health: Are transitory food bugs stowaways or part of the crew? Int. J. Food Microbiol http://dx.doi.org/10.1016/j.ijfoodmicro.2015.03.015

[59] Piel, J. (2002). A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(22), 14002–7.

[60] Portillo, M. L., Vigueras, A. L. (2006). A Review on the Cochineal Species in Mexico, Hosts and Natural Enemies, 249–256.

[61] Ramírez-Cruz, A., Llanderal-Cázares, C., Racotta, R. (2008). Ovariole structure of the cochineal scale insect, *Dactylopius coccus*. *Journal of Insect Science (Online)*, *8*(20), 20.

[62] Ramírez-Puebla, S. T., Rosenblueth, M., Chávez-Moreno, C. K., de Lyra, M. C. C. P., Tecante, A., Martínez-Romero, E. (2010). Molecular phylogeny of the genus *Dactylopius* (Hemiptera: Dactylopiidae) and identification of the symbiotic bacteria. *Environmental Entomology*, *39*(4), 1178–83.

[63] Relman, D. A. (2008) 'Til death do us part': coming to terms with symbiotic relationships. Nature reviews. Microbiol. 6, 721-724.

[64] Ros, V.I., Fleming, V.M., Feil, E.J., Breeuwer, J.A. (2009) How diverse is the genus *Wolbachia*? Multiple-gene sequencing reveals a putatively new *Wolbachia* supergroup recovered from spider mites (Acari: Tetranychidae). App. Environ. Microbiol. 75, 1036–1043.

[65] Rousset, F., Bouchon, D., Pintureau, B., Juchault, P., Solignac, M. (1992). *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. *Proceedings. Biological Sciences / The Royal Society*, *250*(1328), 91–98.

[66] Russell, J. A., Dubilier, N., Rudgers, J. A. (2014). Nature's microbiome: introduction. *Molecular Ecology*, *23*(6), 1225–1237.

[67] Sapp, J. (1994). *Evolution by association. A history of symbiosis* (1st ed.). New York: OXFORD UNIVERSITY PRESS.

[68] Simon, J. C., Boutin, S., Tsuchida, T., Koga, R., Le Gallic, J.-F., Frantz, A., Outreman, Y., Fukatsu, T. (2011). Facultative symbiont infections affect aphid reproduction. *PloS One*, *6*(7), e21831.

[69] Sommer, R. J., Streit, A. (2011). Comparative genetics and genomics of nematodes: genome structure, development, and lifestyle. *Annual Review of Genetics*, *45*, 1–20.

[70] Stintzing, F. C., Carle, R. (2005). Cactus stems (*Opuntia* spp.): a review on their chemistry, technology, and uses. *Molecular Nutrition & Food Research*, *49*(2), 175–94.

[71] Teixeira, L., Ferreira, A., Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in Drosophila melanogaster. *PLoS Biology*, *6*(12), e2.

[72] Tsuchida, T., Koga, R., Fukatsu, T. (2004). Host plant specialization governed by facultative symbiont. *Science (New York, N.Y.)*, *303*(5666), 1989.

[73] Tsucchida, T., Koga, R., Horikawa, M., Tsunoda, T., Maoka, T., Matsumoto, S., Simon, J. C., Fukatsu, T. (2010). Symbiotic bacterium modifies aphid body color. *Science (New York, N.Y.)*, *330*(6007), 1102–4.

[74] Van Dam, A. R., May, B. (2012). A new species of *Dactylopius* Costa (*Dactylopius gracilipilus* sp. nov.) (Hemiptera: Coccoidea: Dactylopiidae) from the Chihuahuan Desert, Texas, U.S.A. *Zootaxa*, *39*(3573), 33–39.

[75] Vavre, F., Fleury, F., Lepetit, D., Fouillet, P., Boulétreau, M. (1999). Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Molecular Biology and Evolution*, *16*(12), 1711–23.

[76] Warnecke, F., Luginbühl, P., Ivanova, N., Ghassemian, M., Richardson, T. H., Stege, J. T., Cayouette, M., McHardy, A. C., Djordjevic, G., Aboushadi, N., Sorek, R., Tringe, S. G., Podar, M., Garcia-Martin, H., Kunin, V., Dalevi, D., Madejska, J., Kirton, E., Platt, D., Szeto, E., Salamov, A., Barry, K., Mikhailova, N., Kyrpides, N. C., Matson, E. G., Ottesen, E. A., Zhang, X., Hernández, M., Murillo, C., Acosta, L. G., Rigoutsos, I., Tamayo, G., Green, B. D., Chang, C., Rubin, E. M., Mathur, E. J., Robertson, D. E., Hugenholtz, P., Leadbetter, J. R. (2007).

Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature*, *450*(7169), 560–5.

[77] Weiss, B. L., Maltz, M., Aksoy, S. (2012). Obligate symbionts activate immune system development in the tsetse fly. *Journal of Immunology (Baltimore, Md. : 1950)*, *188*(7), 3395–403.

[78] Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173:697-703.

[79] Yarza, P., Yilmaz, P., Prusse E., Glöckner, F.O., Ludwig, W., Schleifer, K.H., Whitman, W.B., Euzéby, Amann, R., Roselló-Mora, R. (2014). Uniting the classification of cultures and unclultured batceria and archaea using 16S rRNA gene sequences. Nature Microbiology, 12 (Sep), 635-645.

[80] Yoshida, N., Oeda, K., Watanabe, E., Mikami, T., Fukita, Y., Nishimura, K., Komai, K., Matsuda, K. (2001). Chaperonin turned insect toxin. *Nature*, *411*(May), 44.

[81] Zeh, J. A., Zeh, D. W. (2006). Male-killing *Wolbachia* in a live-bearing arthropod: Brood abortion as a constraint on the spread of a selfish microbe. *Journal of Invertebrate Pathology*, *92*(2006), 33–38.

[82] Zug, R., Hammerstein, P. (2012). Still a Host of Hosts for *Wolbachia* : Analysis of Recent Data Suggests That 40 % of Terrestrial Arthropod Species Are Infected, *7*(6), 7–9.