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DEPARTAMENTO DE BIOTECNOLOGÍA AGRÍCOLA

Creación e identificación molecular de una colección de bacterias de la rizósfera de maíz para el escrutinio de antagonistas a *Fusarium verticillioides*.

JESÚS DAMIÁN CORDERO RAMÍREZ

T E S I S

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PARA OBTENER EL GRADO DE:

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Creación e identificación molecular de una colección de bacterias de la rizósfera de maíz para el escrutinio de antagonistas a *Fusarium verticillioides*

De manera general el tema abarcará los siguientes aspectos:
Creación de una colección de Microorganismos de la rizosfera de maíz
Pruebas *in liquido* para identificar antagonistas a *Fusarium verticillioides*
Pruebas *in planta* para probar potenciales antagonistas a *Fusarium verticillioides*

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3.- El trabajo de investigación base para el desarrollo de la tesina será elaborado por el alumno en:
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Creación e identificación molecular de una colección de bacterias de la rizósfera de maíz para el escrutinio de antagonistas a *Fusarium verticillioides*

Presentada por el alumno:

Cordero	Ramírez	Jesús Damián
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Con registro: A 0 9 0 0 6 5

aspirante de:

Doctorado en Ciencias en Biotecnología

Después de intercambiar opiniones los miembros de la Comisión manifestaron ***APROBAR LA TESIS***, en virtud de que satisface los requisitos señalados por las disposiciones reglamentarias vigentes.

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El presente trabajo se desarrolló en el Departamento de Biotecnología Agrícola del Centro Interdisciplinario de Investigación para el Desarrollo Integral Regional (CIIDIR) Unidad Sinaloa del Instituto Politécnico Nacional (IPN), bajo la dirección del Dr. Ignacio Eduardo Maldonado Mendoza y del Dr. Miguel Ángel Villalobos López (CIBA-Tlaxcala). El presente trabajo fue apoyado económicamente por Fundación Produce Sinaloa (2009-2013), la Secretaria de Investigación y Posgrado del IPN (2009-2013). El alumno Jesús Damián Cordero Ramírez fue apoyado con una beca CONACYT y PIFI-IPN.

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ÍNDICE

1. INTRODUCCIÓN GENERAL

El maíz es el cultivo agrícola más importante de México, tanto desde el punto de vista alimentario, como industrial, político y social. A nivel mundial México ocupa el cuarto lugar en producción de maíz (USDA, 2009)

En el estado de Sinaloa, la producción de maíz aumentó casi hasta el doble en menos de una década, pues de 2,818,000 t. cosechadas en 2001, la producción pasó a 5,368,000 t. en 2008, con rendimientos de productividad por encima de la media nacional. Sinaloa se ubica como el principal estado productor del grano.

Referente al maíz, en el ciclo otoño-invierno de 1990, Sinaloa sembró una superficie de 121,000 ha y para el ciclo 2008-2009, el área sembrada fue de 566,000 ha, con rendimientos de hasta 9.8 t. por ha, para una producción total de 5,236,000 t. En el mismo ciclo se reportaron cerca de 29,716 hectareas siniestradas por distintos problemas; algunas de las cuales pudieron haber sidoafectadas por enfermedades relacionadas al hongo fitopatógeno *Fusarium* spp.

Entre las enfermedades del maíz más importantes están las pudriciones de mazorcas y tallos. A nivel mundial se han reportado como agentes causales de estas pudriciones, a los hongos transmitidos por semilla: *F. proliferatum*, *F. subglutinans* y *F. verticillioides* (White, 2010*)*. En un estudio realizado en México (Morales-Rodriguez *et al.*, 2007) se reportó a siete especies de *Fusarium* asociadas a la pudrición de la mazorca en los Valles Altos de México, al igual que en otras partes del mundo, *F. verticillioides* fue la especie más importante. *F. verticillioides* (Saccardo) Neirenberg (Sinónimo, *Fusarium moniliforme* Sheldon; teleomorfo, *Gibberella moniliformis*) es la especie de hongo más común que infecta a maíz (Munkvold y Desjardins, 1997) causando la pudrición de la

mazorca y del tallo. *Fusarium verticillioides* es el responsable de importantes pérdidas económicas a nivel mundial. La infección del maíz por *F. verticillioides* se puede dar de diferentes maneras, una puede ser sistémica a través de las semillas, en el tallo y raíces, causando la pudrición de la totalidad de la planta (Nelson *et al.*, 1983). Entre su sintomatología podemos incluir: amarillamiento, achaparramiento o enanismo, adelgazamiento del tallo, pudrición de raíz tallo y granos en mazorca, acame por daño en tallo causado por el viento durante el período de secado del cultivo. Es capaz de causar pudrición en tallos y mazorcas, y contaminación de semillas (Duncan y Howard, 2010).Además, este hongo produce un grupo de micotoxinas llamadas fumonisinas, las cuales contaminan el maíz y los productos obtenidos de este cereal. El consumo de estas micotoxinas causa efectos nocivos en los animales y en la salud humana (Shephard *et al.*, 1996).

La especie *F. verticillioides* pertenece a la sección Liseola, específicamente al complejo *G. fujikuroi* (Sawada) Wollenweber, el cual fue subdividido inicialmente en nueve especies: *F. verticillioides, F. sacchari, F. proliferatum, F. subglutinans, F. thapsinum, F. circinatum* y *F. konzum* (Leslie y Summerell, 2006). Actualmente, se considera un número aproximado de 46 especies pertenecientes a este clado (O'Donnell *et al.*, 2000; Marasas *et al.*, 2001; Kvas *et al.*, 2009; Van Hove *et al.*, 2010) . Es un patógeno que se encuentra ampliamente distribuido en las zonas productoras de maíz en el mundo (Leslie y Summerell, 2006); también se ha reportado como patógeno de cultivos como el trigo, arroz, avena y sorgo (Bacon y Nelson, 1994).

Los conidios (2.5-5 x 16-60 µm) hialinos, son curvados cerca de las puntas y con tres a cinco septos. Las microconidias (2-3 x 5-12 µm) son abundantes, unicelulares y arregladas en forma de cadenas (*F. moniliforme* y *F. proliferatum*). Los conidióforos de *F. verticillioides* tiene solamente monofiálides sin ramificar y ramificadas (Nelson *et al.*, 1983; White, 2010).

El ciclo biológico de *F. verticillioides* (*Fv*) es un proceso complejo ya que este hongo es un patógeno no obligado que carece de un hospedero específico. Se puede encontrar ya sea en trigo, sorgo, frijol, soya, algodón, tomate, cacahuate, plátano, pimiento verde, algunos forrajes y en el maíz como su principal hospedero (Bacon y Hinton, 1996; Luna-Olvera, 1998).

Sus distintas fases de vida están conformadas por un estado saprofítico y otro parasítico. Durante la primera etapa, *Fv* obtiene los nutrientes de los tejidos vegetales muertos, produciendo estructuras infectivas para establecer la enfermedad. En su estado parasítico, después de la extensiva colonización intracelular, destruye el tejido a expensas del cual se alimenta, liberando altas concentraciones de fumonisinas (Luna-Olvera, 1998; Oren *et al.*, 2003). La muerte de las plantas de maíz no son muy comunes durante el estado parasítico, pero sí ocurren pérdidas económicas. Además de sobrevivir en restos orgánicos de cultivos anteriores (Cotten y Munkvold, 1998), se reconoce también que se transmite a través de semillas (Bacon *et al.*, 1992). En el suelo, *Fv* regularmente no produce clamidosporas, sino hifas de pared engrosadas que aparentemente prolongan su persistencia (Nelson *et al.*, 1983).

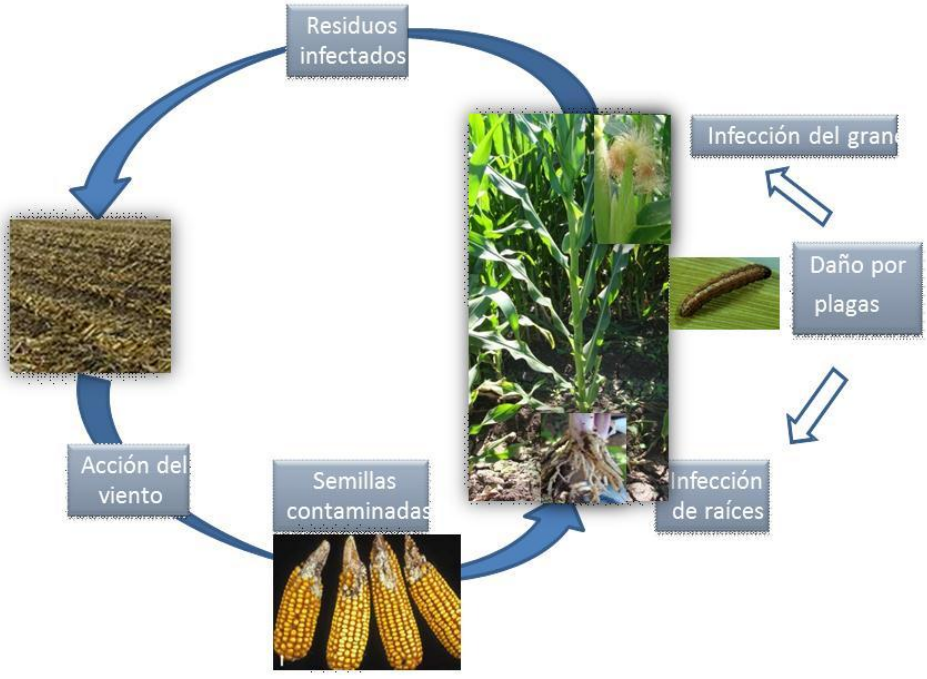

Figura 1. Ciclo de la enfermedad de *Fusarium verticillioides*. Tomado de (Figueroa-López, 2011).

Debido a la gran superficie de siembra que se dedica al maíz en el estado de Sinaloa y a la alta rentabilidad, se propone generar una colección de microorganismos nativos, los cuales ya se encuentran adaptados a las condiciones medioambientales de la región, por lo cual podrían tener un mayor potencial de ser utilizados en el biocontrol de *Fusarium*, en comparación con organismos de biocontrol de formulaciones comerciales; estos últimos comúnmente proceden de otras regiones cuyo ambiente es muy distinto al de Sinaloa.

Con base en estos antecedentes, en el presente trabajo se plantearon los siguientes objetivos:

Objetivo general

Identificación de microorganismos con uso potencial en el biocontrol de *Fusarium verticillioides* en maíz, en el Norte de Sinaloa.

Objetivos específicos

- Obtener e identificar molecularmente una colección de 10,000 microorganismos procariotas (bacterias) cultivables, de rizósfera de maíz.
- Estandarizar la metodología de monitoreo masivo *in vitro* de la colección de microorganismos, para su selección en cuanto a su capacidad antagonista contra *Fv*.
- Seleccionar los mejores antagonistas a *Fv in planta* resultantes de las pruebas *in vitro.*

2.2 CAPÍTULO I

2.1 Interacciones planta-microorganismo

2.1.1 INTRODUCCIÓN

La principal actividad económica de Sinaloa es la agricultura. El Estado cuenta con una superficie agrícola de 1,469,443 has, que representan el 25% de su superficie. El maíz es el cultivo más importante en el estado, tanto por su rentabilidad como por su superficie sembrada. Se estima que la superficie de siembra del ciclo 2008-2009 fue de cerca de 600,000 has con un valor de 15,000 millones de pesos (MDP) (http://www.siap.sagarpa.gob.mx/2009). El maíz es uno de los cultivos más redituables, factor que ha propiciado el monocultivo, lo cual aumenta el riesgo de algunas plagas y enfermedades.

En los últimos años, en Sinaloa se ha incrementado la incidencia de plantas de maíz con pudrición de los tallos, generalmente causada por hongos como *Fusarium* y *Macrophomina*. En un estudio realizado por el Laboratorio de Diagnóstico Fitosanitario de la Junta Local de Sanidad Vegetal del Valle del Fuerte, se encontró que la incidencia de *Fusarium* de las plantas analizadas estaba distribuido en el 70-84% de los lotes de maíz y *Macrophomina* en el 0-1% de los mismos (Quintero-Benítez y Apodaca-Sánchez, 2008) .

El maíz cultivado es hospedante de numerosos microorganismos que pueden producir micotoxinas entre estos *Fv,* el cual puede alojarse en el grano y producir dichas toxinas afectando a la salud humana y animal (Norred, 1993), la cual no ha sido bien caracterizada en nuestro estado y en general, en nuestro país.

2.1.2 ANTECEDENTES

La rizósfera es la zona biológicamente activa alrededor de las raíces de las plantas. Este espacio contiene microorganismos, tales como bacterias y hongos, los cuales interaccionan entre sí, con la planta y con el suelo (Singh *et al.*, 2004). En la rizósfera se genera una serie de interacciones complejas, debido a una actividad biológica intensa y a una transferencia de agua y nutrientes, que pueden resultar benéficas o dañinas a las plantas (Honorato, 2000). El suelo es considerado el hábitat microbiano más importante en la tierra tanto por el número de especies, como por el tamaño de las comunidades, siendo las bacterias el grupo más abundante de microorganismos del suelo (Gans, 2005). En la rizósfera abundan una amplia gama de compuestos orgánicos tales como exudados de raíces de bajo peso molecular, secreciones, mucigeles, lisados celulares y metabolitos. En consecuencia las raíces actúan como una fuente de compuestos de carbono, y con ello, la densidad de las poblaciones de microorganismos es considerablemente más alta en la rizósfera, en comparación con zonas del suelo cercanas a la raíz, pero no tan inmediatas (Lynch, 1990; Bolwerk *et al.*, 2003; Martínez-Álvarez, 2003). La acumulación total de estos componentes asociados a raíz en la rizósfera se le denomina rizodeposición. La rizodeposición es un proceso dinámico, el cual es regulado y varía, dependiendo de la especie de planta y cultivar (Marilley y Aragno, 1999; Faure *et al.*, 2008). Aun cuando esta rizodeposición representa un costo significativo de carbono para las plantas y la magnitud de los fotosintatos secretados por las raíces varía de acuerdo al tipo de suelo, edad y estado fisiológico de la planta, así como de la disponibilidad de nutrientes (Bais *et al.*, 2006). Sin embargo, este fenómeno es relevante desde el punto de vista ecológico, pues además de contribuir a la heterogeneidad del suelo (Marilley y Aragno, 1999), también puede detener el crecimiento de un organismo patógeno para la planta o bien atraer a otro benéfico (Morris *et al.*, 1998; Chin-A-Woeng *et al.*, 2000; Kamilova *et al.*, 2006). Los microorganismos asociados a las raíces, desempeñan funciones de gran importancia en relación con procesos de edafogénesis (Wright y Upadhyaya, 1998); en los ciclos biogeoquímicos de

elementos como el carbono, el nitrógeno, oxígeno, el fósforo y el azufre (Madigan *et al.*, 2004); influyen en la fertilidad de las plantas (O'Donell *et al.*, 2001) y promueven la protección vegetal frente a patógenos (Whipps, 2001). Los microorganismos interactúan con los tejidos y las células con diferentes grados de dependencia, lo cual les ha permitido desarrollar varias estrategias para adaptarse al entorno de la planta (Trivedi *et al.*, 2010a).

En estudios previos se ha reportado que la estructura de las comunidades bacterianas del suelo puede ser modificada por el uso del suelo (da C Jesus *et al.*, 2009; Nacke *et al.*, 2011), especie vegetal (Marilley *et al.*, 1998; Wieland *et al.*, 2001), tipo de suelo (Girvan *et al.*, 2003), textura del suelo (Sessitsch *et al.*, 2001), disponibilidad de nitrógeno (Frey *et al.*, 2004), y por la presencia y asociación de patógenos en las raíces de las plantas (Trivedi *et al.*, 2010a; Mendes *et al.*, 2011) .

Aun cuando se conoce que en la rizósfera existen microorganismos benéficos para las plantas, los cuales les permiten disminuir los síntomas de las enfermedades y mejorar el rendimiento, a la fecha solo pocos trabajos han examinado la influencia de los fitopatógenos en la diversidad microbiana de las asociaciones planta-bacteria (Mazzola y Cook, 1991; McSpadden Gardener y Weller, 2001; Yang *et al.*, 2001; Araujo *et al.*, 2002; Reiter *et al.*, 2002; Filion *et al.*, 2004; Trivedi *et al.*, 2010a). En el presente trabajo se estudiaron las comunidades bacterianas cultivables a partir de una colección de aislados bacterianos obtenidos de la rizósfera de maíz de plantas enfermas y sanas de la zona Norte del estado de Sinaloa, para (i) identificar aquellas comunidades bacterianas presentes en plantas sanas que pudieran estar influyendo en la disminución de síntomas ocasionados por *Fv*, o bien, aquellas comunidades que pudieran causar un efecto sinergista con el patógeno, y (iii) seleccionar aquellas bacterias que pudieran ser utilizadas en el desarrollo de agentes de biocontrol para el hongo *Fv*.

La siguiente sección presentada como anexo 1 responde al primer objetivo planteado en el trabajo: Creación e identificación molecular de una colección de

bacterias de la rizósfera de maíz para el escrutinio de antagonistas a *Fusarium verticillioides*.

2.1.3 ANEXO I Sometido en la revista Plant and Soil

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Short Running Title: Culturable bacteria from maize rhizosphere

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Identification of culturable bacteria from the rhizosphere of *Fusarium verticillioides* symptomatic and asymptomatic maize plants

Running title: Culturable bacteria from the maize rhizosphere

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Abbreviations: SERR: stalk, ear, and root rot of maize; *Fv*: *Fusarium verticilliodes.*

ABSTRACT

Background and Aims

The stalk, ear, and root rot (SERR) of maize caused by *Fusarium verticillioides* (*Fv*) severely impacts crop production in tropical and subtropical regions. This work explores the populations of culturable bacterial genera known for antagonism against *Fv*, including *Bacillus* and *Pseudomonas*, and their relationship to SERR symptoms in agricultural fields.

Methods

Rhizosphere samples were taken from SERR symptomatic or asymptomatic maize plants in five maize field sites, and a culturable bacterial collection enriched in Firmicutes and Proteobacteria was created. Physicochemical soil analysis was performed on each soil sample. 16S rDNA sequences were analyzed and grouped into Operational Taxonomic Units (OTUs), which enabled examining OTU number and distribution in plants with different infection statuses.

Results

We identified 7, 077 bacteria, which were classified into 185 non-singleton OTUs. Depending on the plant health status, some OTUs were statistically different in abundance, whereas others were exclusively present in SERR symptomatic (38 OTUs) or asymptomatic plants (36 OTUs). Soil physicochemical properties were similar between symptomatic and asymptomatic plants, and had little impact on the distribution of identified OTUs.

Conclusions

After analyzing bacterial OTU abundance we confirmed that specific populations from *Bacillus* and γ-Proteobacteria (mainly *Pseudomonas* and *Enterobacter*) were affected by *Fv* infection status. Fluctuations in their populations within the maize rhizosphere suggest their possible involvement in allowing or preventing SERR development in maize, under field conditions.

Keywords: *Fusarium verticillioides*; community structure; biocontrol agents; maize; bacterial populations

INTRODUCTION

Plant roots provide a nutrient-rich environment for a large number of soil microorganisms. The rhizosphere, a zone in close proximity with the root surface, typically contains 10 to 100 times more microorganisms per gram than bulk soil (Haas *et al.*, 2002). The organisms harbored by the rhizosphere can have a neutral effect on the plant, or even deleterious or beneficial effects (Raaijmakers *et al.*, 2008). Different factors, such as land use (da C Jesus *et al.*, 2009; Nacke *et al.*, 2011), soil type (Girvan *et al.*, 2003), soil texture (Sessitsch *et al.*, 2001), pH (Bååth y Anderson, 2003; Lauber *et al.*, 2009), nitrogen availability (Frey *et al.*, 2004) and plant species (Wieland *et al.*, 2001) affect the bacterial community structure. Moreover, diversity and stability of plant-associated bacterial communities influence soil and plant quality, as well as ecosystem sustainability (Kennedy y Smith, 1995; Lukow *et al.*, 2000; Nannipieri *et al.*, 2003). Several studies have examined the effect of phytopathogens on the microbial diversity of plant-associated bacteria (Mazzola y Cook, 1991; McSpadden Gardener y Weller, 2001; Yang *et al.*, 2001; Araujo *et al.*, 2002; Reiter *et al.*, 2002; Filion *et al.*, 2004; Trivedi *et al.*, 2010b; Mendes *et al.*, 2011). These reports show that the presence of plant pathogens influences population dynamics in the rhizosphere, and in some cases certain groups of bacteria might affect disease development (Yang *et al.*, 2001; Mendes *et al.*, 2011). The study of plant-associated microbiota can facilitate and improve several biotechnological applications, such as the biological control of plant pathogens, plant growth-promotion, and isolation of bioactive compounds (Emmert y Handelsman, 1999; Bloemberg y Lugtenberg, 2001).

Fusarium verticillioides (*Fv*) constantly accompanies maize plants (*Zea mays*) and seeds (Munkvold *et al.*, 1997). This pathogen is among the most commonly found fungi that colonize symptomless maize plants. In maize, the fungus causes stalk, ear and root rot (SERR), as well as seedling blight (Marjana *et al.*, 2003). In many cases, the presence of *Fv* is ignored because it does not cause any visible damage. Symptomless infections can exist throughout the plant, and seed-transmitted strains of the fungus can systemically develop to infect the kernels (Kedera *et al.*, 1992; Munkvold *et al.*, 1997). In recent years, the monoculture of maize in Sinaloa, Mexico has provoked an increased incidence of fungal diseases like SERR. For example, one field study has found a 70-84% prevalence of *Fusarium* in maize fields (Quintero-Benítez y Apodaca-Sánchez, 2008). At the same time, Sinaloa benefits from a massive cultivated area (0.5-0.6 million

ha yearly) and a highly technified crop system, making this region an excellent model to study *Fv* infection of maize fields in tropical areas under an irrigation regime.

A more complete understanding of the microbial ecology and diversity associated with the maize rhizosphere could improve plant health in field crops, reduce our dependence on chemical pesticides used in agriculture, and develop efficient biological control strategies (Smith *et al.*, 1999; Filion *et al.*, 2004). Therefore, it is important to characterize the indigenous microbial communities naturally associated with maize root systems, to identify potential biocontrol agents (Cavaglieri *et al.*, 2009; Mendes *et al.*, 2011). To address this, recent studies have focused on characterizing rhizospheric maize bacterial communities using non-cultivation approaches and employing new sequencing technologies (Pereira *et al.*, 2011a). However, there is a drawback to metagenomic studies performed on DNA soil samples: dormancy allows bacteria to persist during unfavorable conditions, and recent surveys estimate that over 80% of the bacterial cells in the soil are dormant (Lennon y Jones, 2011). Moreover, the community of physiologically active bacteria within the soil is distinct from those that are dormant (Lennon y Jones, 2011). As a result, estimates of bacterial composition using standard DNA extractions from soil may not provide measurements that reflect the active players in the plant-microbe interaction, potentially obscuring field attempts to identify the agents of microbial control (Bulgarelli *et al.*, 2013).

In this work, we chose to examine the abundance of culturable bacterial populations from the maize rhizosphere of SERR symptomatic and asymptomatic plants. We used a cultivation-based approach designed to specifically enrich for bacterial genera such as *Bacillus* and *Pseudomonas*, previously reported to be *in vitro* growth inhibitors of *Fv* (Cordero-Ramírez *et al.*, 2012a). Our aim was to identify changes in culturable bacterial populations that could be indicators of SERR disease development. This work will improve our understanding of which targeted bacterial soil populations are found in SERR symptomatic and asymptomatic maize plants under agricultural conditions. Our results will also be an invaluable resource to future studies, in terms of properly designing field application schemes for bacterial biocontrol agents.

MATERIALS AND METHODS

Sample collection

A total of fifty maize rhizosphere samples were collected from five locations. Plants were sampled in five paired groups, each pair consisting of one SERR symptomatic plant and one asymptomatic plant. Pairs were taken as adjacent plants from a single row in the field. The field sampling took place in five different locations in Sinaloa: I) Serrano; II) Alhuey; III) 18 de Diciembre; IV) Casa Blanca; and V) La Trinidad. Samples were collected in February and March of 2009. 3-4 kg of soil were removed from the stem base of each plant with a shovel at a depth of 0-40 cm, leaving the root system mixed with the soil. These fields showed symptoms of plant damage by the fungus *F. verticillioides* (Figure 1). Each of the five sampling points differed by planting day and by maize hybrids (Table S1). Microbiological analyses were conducted to confirm SERR symptomatology, and *Fv* was only isolated from SERR symptomatic plants in selective media. ITS rDNA sequencing was used to confirm the identity of the fungal isolates as *Fv*.

Sample processing and physicochemical soil analysis

Root tissue was separated from bulk soil by vigorous shaking, which left soil particles strongly bound to the root. These soil particles were carefully collected to ensure that only rhizospheric soil was taken for bacterial isolation. Each rhizospheric soil sample was air dried for three to five days and passed through a 1 mm mesh screen to eliminate large particles. The five samples from each condition and location were homogenized together and stored at room temperature (O'Brien *et al.*, 2005). To isolate microorganisms, we first took a sub-sample to prepare an aqueous homogenate. From this, serial dilutions (containing organisms) were plated, using 100 μL per dilution. Four different culture media were prepared in 100 mm-diameter Petri dishes to enrich for specific taxonomic groups. Thus, Luria Bertani (LB) medium was used for enrichment of *Bacillus* isolates (Cavaglieri *et al.*, 2005b); Actinomycetes Isolation Agar [AIA] was used for Actinomycetes isolates (Bressan, 2003a; González *et al.*, 2005; Bressan y Figueiredo, 2007); King B Agar (KBA) was used for *Pseudomonas* (Broek *et al.*, 2003; Cavaglieri *et al.*, 2004); and Man, Rogosa and Sharpe (MRS) medium was used for lactic acid bacteria. Plates were grown at 25°C.

A bulk soil sub-sample (500 g) was used for nutrient and physicochemical soil analyses (texture, NPK, pH, and organic matter). Texture was determined based on soil texture classification by particle size distributions (USDA), while phosphate was analyzed according to Olsen *et al.* (1954), and organic matter according to Walker and Black (1934). Each soil sub-sample was analyzed separately for soil physicochemical properties, except for texture in which all samples were pooled together (Table S1).

Microorganism collection and viability test

Colonies were taken from LB, KBA and MRS media after 24 hours growth, and from AIA medium after 48-72 hours. This procedure allows for selecting a subset of fast-growing organisms highly enriched for the two main bacterial genera of our interest: *Bacillus* and *Pseudomonas*, which have been previously described as *Fv* antagonists (Cordero-Ramírez *et al.*, 2012b). To generate the maize rhizospheric bacterial collection, approximately 288 isolates were "picked" from each specific culture medium, yielding 1,152 isolates from each of the ten composite rhizospheric samples; the complete collection therefore contained 11,520 isolates. The isolates were cryopreserved in 96-well plates at -70°C, using 200 µL of LB containing 15% glycerol (Pasarell y McGinnis, 1992). Three replicates of the collection were prepared and each one was stored in a different -70°C freezer. Frozen stocks were made from each isolate and were grown at 25°C and 200 rpm in 2 mL 96-well plates containing 1.5 mL liquid medium for either 24 hours (LB, KBA and MRS media), or 72 hours (AIA medium). The isolate was considered nonviable if no visible growth was observed after thawing. The plates containing bacterial pellets from viable isolates were stored at -70°C until processed for DNA extraction.

Bacterial DNA extraction and amplification of 16S rDNA

Bacterial DNA was extracted with the DNeasy® Blood & Tissue Kit (Qiagen, CA, USA), using a Qiacube robotic platform (Qiagen, CA, USA) as described by the manufacturer. The pellets were dissolved in 100 µL of elution buffer. The bacterial primers F2C (5'- AGAGTTTGATCATGGCTC -3') and C (5'- ACGGGCGGTGTGTAC -3') (Shi *et al.*, 1997) were used to amplify 16S rDNA. The reactions were carried

out in a Qiagility robotic platform (Qiagen, CA, USA) in 96-well plates. The 25 µL PCR mixture contained 10 ng of DNA template, 1X reaction buffer, 10 pmol of each primer, 10 µM of each deoxynucleoside triphosphate (dNTP), and 1 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). For product amplification, the DNA templates were first heated in an initial denaturation step at 95°C for 4 min. The subsequent cycles consisted of a 1 min denaturation step at 95°C, a 1 min annealing step at 60°C, and a 1.5 min extension step at 72°C. The program concluded after 32 cycles with a final 5 min extension step at 72°C. The PCR was performed using a MyCycler thermal cycler (BioRad, CA, USA). Products were visualized by 1% agarose gel electrophoresis in 0.5 X Tris-acetate-EDTA (Gao *et al.*) buffer and stained with ethidium bromide, to verify product size. PCR products were purified with a QIAquick® PCR Purification kit (Qiagen, CA, USA) and quantitated using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Scientific). The U1 primer (5' CCAGCAGCCGCGGTAATACG 3') (Lu *et al.*, 2000) internal to the F2C/C amplified PCR product was used for sequencing with an ABI 3730 XL automated sequencer at the National Laboratory of Genomics (LANGEBIO, Irapuato, Mexico).

Sequence analysis

High quality sequences were first examined for the presence of possible chimeric sequences with the CHIMERA_CHECK program, available from the Ribosomal Database Project II (RDP II) website. All chimeric sequences were discarded. Next, the microorganism diversity associated with symptomatic and asymptomatic maize rhizosphere samples was investigated by performing a rarefaction analysis. For this, sequences were aligned with the Clustal-W program (Thompson *et al.*, 1994). The distance matrix was calculated with the Dnadist Phylip Program (v. 3.69), using the Jukes-Cantor substitution model. The sequences were grouped into organizational taxonomic units (OTUs) with the Cluster program using the Average Neighbour algorithm from the Mothur program v. 1.20.1 (http://www.mothur.org) (Schloss *et al.*, 2009). The OTUs were defined as a group of sequences sharing at least 97% pairwise similarity. All singletons were removed, to avoid any bias from minimally represented sequences. A Shannon index was calculated (Shannon, 1984), and all resulting DNA sequences were subsequently identified by comparing them against the GenBank/EMBL/DDBJ database, using the BLASTN search algorithm

(http://blast.ncbi.nlm.nih.gov). The sequences were then compared on the basis of identity percentage, E-value and Match score, using the default parameters from the RDP seq match tool (http://rdp.cme.msu.edu/). All 7,077 16S rDNA gene sequences from each isolate were deposited in GenBank, with accession numbers JQ829081 through JQ836199.

Statistical analyses

Chi-square analyses were conducted for each OTU using PROC FREQ, to determine the effects of sampling location and infection status of the plant on the bacterial population distributions. Physicochemical soil data were evaluated with the statistical package STATISTICA 7.0, using one way analysis of variance contrasting SERR symptomatic vs. asymptomatic soil samples.

RESULTS

Bacterial collection and identification

The bacterial collection from both symptomatic and asymptomatic maize plants and from all sampling points comprises 11,520 isolates. Two months after freezing and thawing, isolates were observed to exhibit 95% survival efficiency, yielding a new total of 10,944 isolates. From this collection, a 16S rDNA high quality sequence was obtained for each isolate. A total of 7,077 isolates were identified when compared against both RDP and GenBank databases.

Rarefaction analysis

The level of bacterial diversity represented in this collection was estimated by rarefaction analysis (Figure 2). This analysis consisted of aligning the 7,077 16S rDNA sequences obtained and grouping them into Operational Taxonomic Units (OTUs) on the basis of different sequence dissimilarity cut-offs. By using this

procedure with a 3% sequence dissimilarity criterion, all sequences were grouped into 689 OTUs, 504 singletons (containing only one sequence per OTU and thus eliminated to avoid bias in further analysis) and 185 non-singleton OTUs. Rarefaction analysis revealed that the species sampling effort curves calculated with 95 and 97% sequence identity have a positive slope with no evidence of approaching saturation (Figure 2). This indicates that although a substantial number of isolates were sequenced, the bacterial diversity in these samples is much greater, and a larger sequencing effort is needed to cover their entire diversity. This was not unexpected, since the aim of this work was not to study total bacterial diversity, but rather to isolate culturable bacteria from four different media, in a search biased to enrich for *Bacillus* and *Pseudomonas*.

Classification of culturable bacteria from the rhizosphere of symptomatic and asymptomatic maize plants

Identification at the genus level, using RDP and GenBank databases, was successful for most isolates; however, species identification is very limited when only 16S ribosomal DNA sequences are used. Nevertheless, by assigning putative taxonomic identities to each OTU we identified 19 genera and 44 species from the bacterial communities of the maize rhizosphere (Tables S2, S3 and S4).

We identified bacteria in four different phyla: Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes. Firmicutes was the most abundant phylum (in terms of number of isolates per phyla), with most isolates belonging to the genus *Bacillus*. The second most abundant phylum was Proteobacteria, with most isolates assigned to the γ-Proteobacteria class. The Actinobacteria and Bacteroidetes phyla were represented by <1% of the classified isolates (Table S2).

Firmicutes and Proteobacteria had the greatest putative species richness in the culturable bacterial populations studied from the maize rhizosphere; Proteobacteria was the most diverse phylum, represented by ten different genera (Table S2). The most abundant class was γ-Proteobacteria, followed by α- and β-Proteobacteria. The γ-Proteobacteria class was represented mainly by the genera *Pseudomonas* and *Enterobacter*, followed by *Pantoea*, *Stenotrophomonas*, *Acinetobacter*, *Aeromonas*, *Klebsiella* and *Cronobacter*. The β-Proteobacteria class was solely represented by the genus *Massilia*. The α-Proteobacteria class was represented by the genus *Rhizobium* (Table S2). At the species level, the most highly represented

species were *E. cloacae, P. fluorescens* and *P. putida*, with at least 100 different isolates per species. This phylum was also represented by seven other putative species (Table S3).

Firmicutes was the second most diverse phylum, with six genera represented by 146 out of 185 OTUs. As noted earlier, the Firmicutes isolates belong mainly to *Bacillus* spp. (represented by up to 36 different OTUs). These include *B. cereus, B. firmus, B. megaterium, B. subtilis* and other less abundant genera such as *Lysinibacillus*, *Paenibacillus*, *Terribacillus*, *Staphylococcus* and *Brevibacillus* (Tables S2 and S3). Among these species, six were individually represented by more than 100 isolates each one.
The least diverse groups are the Actinobacteria and Bacteroidetes phyla. In Actinobacteria we identified the genera *Arthrobacter* and *Sinomonas*, and in Bacteroidetes we identified the genus *Sphingobacterium* (Table S2). These analyses of genera representation confirm that Firmicutes and Proteobacteria were the most diverse culturable phyla isolated from the maize rhizosphere, using our approach.

Diversity and physicochemical soil characteristics

Microbiological analysis confirmed the presence of *Fv* (by isolating and sequencing this fungal pathogen) in symptomatic root sections ; by contrast, *Fv* was not present in asymptomatic root sections (unpublished data). The sheer number of OTUs was also used as an indicator of diversity, to obtain more information about the bacterial diversity found at each sampling point. These data were used to calculate the Shannon index for each sampling site. This index was slightly higher in asymptomatic plants than in SERR symptomatic plants in two sites (I and V), whereas the opposite was observed at the other three sites (II, III, and IV) (Table 1). Furthermore, these three sampling points (II, III, and IV) displayed a greater number of OTUs in SERR symptomatic plant rhizospheres, as compared to asymptomatic plant rhizospheres. By contrast, the other two sampling points (I and V) had a greater number of OTUs in the rhizosphere of SERR asymptomatic plants (Figure 3). These results indicate that OTU diversity did not consistently associate with plant health status. No significant differences were observed for any of the soil chemical or physical parameters evaluated when comparing symptomatic versus asymptomatic plants at each sampling point. This suggests for the most part that these factors cannot explain differences in bacterial populations found between SERR symptomatic and

asymptomatic plants (Table 1). The only exception to this is sampling sites I and II, where significant changes in pH were detected.

OTUs with differential distribution in SERR symptomatic or asymptomatic infected maize rhizospheres

When comparing the distribution of OTUs across all five locations and two infection statuses, we found that 36 OTUs specifically associated with the rhizosphere of asymptomatic plants, 38 OTUs with the rhizosphere of SERR symptomatic plants, and 111 OTUs associated with both types of rhizospheres (Figure 3). In addition, the distribution in SERR symptomatic and asymptomatic plant rhizospheres reveals that *Bacillus* (27 OTUs) and *Pseudomonas* (6 OTUs) were mainly present in asymptomatic plants. Although this was also true for SERR symptomatic plants (with 32 OTUs from *Bacillus* and 2 from *Pseudomonas*), fewer *Pseudomonas* OTUs were observed in this condition. Thus, *Pseudomonas* OTUs were mainly associated with healthy looking asymptomatic plants.

Several interesting distribution patterns emerge when taking into account the OTU distribution of both SERR asymptomatic and symptomatic plants at each sampling site. Eighteen out of the 36 OTUs found only in asymptomatic plant rhizospheres were from site I. These OTUs represent the genera *Bacillus* (10), *Pseudomonas* (6), *Paenibacillus* (1) and *Enterobacter* (1). Six other OTUs were found uniquely in one location, comprising 1 in site II (*Bacillus*), 2 in site III (*Bacillus*), 1 in site IV (*Bacillus*), and 2 in site V (1 *Acinetobacter* and 1 *Bacillus*). Each of the 12 remaining *Bacillus* OTUs were found in more than one site (Table 1).

The 38 OTUs that were only found in the rhizosphere of SERR symptomatic plants showed a different distribution. Two OTUs (*Cronobacter* and *Bacillus*) were found in site I, 1 in site II (*Pseudomonas*), 4 in site III (2 *Bacillus*, 1 *Aeromonas* and 1 *Pseudomonas*), 3 in site IV (*Bacillus*), and 3 in site V (1 *Bacillus* and 2 *Lysinibacillus*). Each of the 25 other *Bacillus* OTUs were found in more than one site (Table 1).
In some cases, several OTUs were differentially distributed in one location according to the infection status, although not for other locations. Therefore, the OTUs that were present in at least two different locations were statistically analyzed by chi-squared test to determine whether they were affected by location/infection status, or if they were randomly distributed. We thus identified twenty-seven OTUs affected by location, of which

nine OTUs were also affected by infection status. Another nine OTUs were only affected by infection status: OTUs 1, 2, 7, 22, 82, 94 were predominant in SERR symptomatic rhizospheres, whereas OTUs 17, 36, 383 were predominant in asymptomatic maize rhizospheres (Table 2).

DISCUSSION

To the best of our knowledge, this is the first study to analyze culturable bacterial communities from the maize rhizosphere, with respect to SERR symptoms caused by *Fusarium verticillioides.* The effect of a pathogen on native microbial communities has previously been studied in diseases affecting other crops, such as citrus (Araujo *et al.*, 2002; Trivedi *et al.*, 2010b), conifers (Filion *et al.*, 2004), wheat (Mazzola y Cook, 1991; McSpadden Gardener y Weller, 2001), potato (Reiter *et al.*, 2002), and avocado (Yang *et al.*, 2001). These studies demonstrate that phytopathogens affect the population dynamics of endophytic and rhizospheric bacterial populations, and that some bacterial populations may facilitate the suppression of disease. Our sampling of culturable bacteria from maize rhizospheres presented in this work indicates that SERR symptoms in maize plants produce small but significant and distinguishable changes, at least at the bacterial population level at every single sampling point. These changes include a modified abundance of specific genera, as well as the presence of specific OTUs according to maize health status. This also demonstrates, despite the health status and the contrasting levels of symptoms in maize plants (Figure 1), that the overall diversity of culturable bacteria is maintained at the same level.

Several bacterial genera have been observed in greater abundance in the rhizosphere of wheat plants when they are afflicted by take-all disease, as compared to healthy plants (McSpadden Gardener y Weller, 2001).

In our study, the composition of culturable bacterial populations from the maize rhizosphere displayed little correlation with plant health status at the OTU/species level (Tables 1, 2 and S4). Most of the changing bacterial populations belonged to the two most abundantly represented phyla in the bacterial collection, Firmicutes and Proteobacteria. Interestingly, bacterial populations associated with the rhizosphere of either SERR symptomatic or asymptomatic maize differ in both number and composition (Figure 3; Table

S4) for each sampled maize field point. A clear distinction was observed between rhizospheres of either SERR symptomatic or asymptomatic plants, in which specific OTUs were found to be uniquely present in either SERR symptomatic or asymptomatic plants (Figure 3, Table 1), and their abundance correlated with symptomatic or asymptomatic plants (Table 2). These findings suggest that plant disease in SERR symptomatic plants may play a role in the establishment or replacement of specific bacterial populations in the host rhizosphere, regardless of location.

This study did not aim to decipher overall maize rhizospheric bacterial diversity, but rather to clarify how specific culturable bacterial populations, from genera reported as antagonistic to *Fv*, change within the rhizosphere of SERR symptomatic or asymptomatic plants. Nevertheless, it is important to consider that the rarefaction analysis clearly shows that our sampling could not cover the entire diversity of bacterial populations (Figure 2), suggesting that the species richness is actually much greater than what can be determined from our sampling and sequencing techniques. The level of species diversity in culturable bacterial populations presented here according to the Shannon index (2.20-3.29; Table 1) could be influenced by our biased isolation method to enrich for *Bacillus* and *Pseudomonas*. Various other factors could also influence these results, including: difficulty in dislodging bacteria or spores from soil particles or biofilms; growth conditions (temperature, pH and light); the inability to culture a large number of species with current *in vitro* techniques; the possibility of colony-colony inhibition or colony spreading; and the use of a growth medium that may favor microorganisms with faster growing rates and that can produce large numbers of spores (Kirk *et al.*, 2004).

Our group previously found, while studying tomato (Cordero-Ramírez *et al.*, 2012b) and *Datura stramonium* (López-Rivera, 2011) rhizospheres, that Firmicutes rhizospheric species grow very actively in LB medium, and that they are highly represented in culturable bacterial populations. Similar results were obtained when 16S rDNA sequencing surveys were used as a culture-free method to assess communities in these species with Firmicutes as the dominant phyla (López-Rivera, 2011; Cordero-Ramírez *et al.*, 2012b). This indicates that both methods could produce results that are representative of the bacterial populations for community assessment in maize rhizosphere, suggesting that culturable bacterial populations could be similar to those found by culture-free methods. Nonetheless, this possibility still needs to be experimentally demonstrated using new generation sequencing techniques.

Among the phyla identified in our study Proteobacteria, Firmicutes and Actinobacteria were previously identified in a metagenomic study as the most changing taxa in the sugar beet rhizosphere associated with disease suppression of *Rhizoctonia solani* (Mendes *et al.*, 2011). In agreement with our findings (Tables S2 and S3), Firmicutes and Proteobacteria have also been reported as the most abundant and diverse phyla in the maize rhizosphere of 20-day old maize seedlings (Pereira *et al.*, 2009; Pereira *et al.*, 2011a). In these reports, *Bacillus* and *Pseudomonas* were observed to be the major community components of the maize rhizosphere, using either culture-dependent or independent methods.

Although the presence of specific OTUs is significantly affected in sampling sites, most of the bacterial populations did not show significant changes in their abundance between sampling sites (Tables 2 and S4). As physicochemical soil characteristics did not show any significant differences when comparing symptomatic and asymptomatic bulk soil samples (Table S1), these characteristics cannot explain SERR symptoms. Instead, it is likely that microbiota changes associated with the disease influence the Fv infections status on maize plants.

Several putative *Bacillus* species from Firmicutes (the most abundant bacterial phylum in the maize rhizosphere) were the most prominent bacterial populations in this study. Members of this genus have been well-described as either plant growth-promoting rhizobacteria (PGPR) or as biocontrol agents (Nagórska *et al.*, 2007). For example, *Bacillus cereus* increased grain yield by 43.8% in maize (cv. GS-2) following seed bacterization, as compared to a non-inoculated control (Tilak y Reddy, 2006). *In vitro* testing of *Bacillus subtilis* in maize roots and kernels inhibited both *Fv* growth and the production of fumonisin B1 (Cavaglieri et al. 2004). *Bacillus megaterium* isolated from the maize rhizosphere has been reported to promote growth and development of *Phaseolus vulgaris* and *Arabidopsis thaliana* (López-Bucio *et al.*, 2007). *Bacillus thuringiensis* is used as a biocontrol agent of phytopathogens (Lucon *et al.*, 2010). In the present work, we determined that Firmicutes was the most abundant phylum, and had the most relevance to *Fv* infection status on the maize rhizosphere (Tables 1 and 2). Our results clearly show that Firmicutes OTUs associated with both SERR symptomatic and asymptomatic plants, thus we can only suggest that some of these OTUs may have a role in disease avoidance or symptom development.

The second most abundant phylum that we observed in the maize rhizosphere was Proteobacteria, in which the most predominant genera were *Enterobacter* and *Pseudomonas*. *Enterobacter* isolates have been

isolated from roots of maize (Hinton y Bacon, 1995), wheat (Kämpfer *et al.*, 2005) and *Lolium perenne* (Shoebitz *et al.*, 2009). In a previous study, *Enterobacter cloacae* displayed an endophytic distribution within maize stem and leaf tissues, and exhibited an antagonistic effect against *Fv* (Hinton y Bacon, 1995). In our study, this species was represented by 399 sequences in OTU 22 and was distributed across three locations (I, II, and III), with a significantly higher population in SERR symptomatic plants (Table 2). A possible scenario to explain these observations could be that disease establishment in the host plant causes a shift in bacterial populations; this could then allow an increase in specific bacterial groups, especially endophytic ones. This could also possibly result in a significant increase in biocontrol activity, as a line of defense against the development of the fungal pathogen.

Pseudomonas are one of the most dynamic groups at the population level as well as the most abundant rhizospheric bacteria, and they have been previously described as biocontrol agents (Hebbar *et al.*, 1992b; Höfte y Altier, 2010; Mendes *et al.*, 2011). Bacteria belonging to Pseudomonadaceae have been isolated from suppressive soils and exhibited antagonism against *Rhizoctonia solani* in sugar beet roots, and were more abundant in suppressive soils than in disease-conducive soils (Mendes *et al.*, 2011). In addition, Costa et al. (2006) associated the functional and structural diversity of *Pseudomonas* by matching dominant ribotypes (DGGE) of *Pseudomonas* spp. in the maize rhizosphere with PCR-DGGE fingerprints of bacterial isolates that display an antagonistic potential against the phytopathogenic bacteria *Ralstonia solanacearum*. In this work, two abundant OTUs (36 and 383), putatively identified as *P. putida* and *P. fluorescens*, were significantly more abundant in asymptomatic than in SERR symptomatic maize plants (Table 2). *Pseudomonas* populations may show an increase in plants that do not develop any SERR symptoms in response to the fungal phytopathogen.

None of the OTUs that were found exclusively in SERR symptomatic or asymptomatic plants were identified across all five sampling points. However, some OTUs (primarily from *Bacillus* and *Pseudomonas*) were mainly found associated to either symptomatic or asymptomatic conditions, in up to three sampling points (Table 1). Other OTUs that were found only in symptomatic plants showed homology to *Aeromonas* (site III), *Cronobacter* (site I) and a member of the Bacteroidetes phylum, *Sphingobacterium* (sites II and III; Table 1). It is unclear whether isolates from these genera participate in the development of SERR symptoms, or if they are a result of *Fv* infection of the plant. Nevertheless, they may also have a role in regulating the

plant health status. The genera *Aeromonas* (Karagöz *et al.*, 2012) and *Cronobacter* (Schmid *et al.*, 2009) were isolated previously from grapevine rhizosphere, and *Sphingobacterium* was isolated from maize rhizosphere (Mehnaz *et al.*, 2007). To the extent that we are aware, a role for the members of these genera in plant disease development has not yet been described. Nevertheless, we cannot rule out the possibility that these genera act synergistically or individually to promote *Fv* infection in the maize plant.

Root exudates may undergo change during disease development, producing concomitant shifts in bacterial communities and possibly even altering rhizospheric pH (Bais *et al.*, 2006). In our study, strong changes in pH were not detected in SERR symptomatic plant rhizospheres (as compared to asymptomatic rhizospheres); in fact, pH increases were only observed in sites I and II (0.6 and 0.4 units, respectively), in the rhizosphere of SERR symptomatic plants (Table S1). Interestingly, a decrease in the number of OTUs was associated with the development of SERR symptoms in site I (Figure 3). Eighteen out of 36 OTUs from this site were only associated with asymptomatic plant rhizospheres (Table 1), suggesting that a decrease in bacterial populations of specific OTUs belonging to *Enterobacter*, *Bacillus* and *Pseudomonas* could lead to disease symptoms.

Based on the results obtained here and in previous work (McSpadden Gardener y Weller, 2001; Mendes *et al.*, 2011), we propose that disease suppression cannot simply be ascribed to a single bacterial taxon or group; instead, it is more likely governed by microbial consortia. This study provides insight into the culturable bacterial communities from the maize rhizosphere, and how the presence of SERR caused by *Fv* leads to changes in abundance and diversity of bacterial populations. Since we did not reach population sampling saturation in this study (Figure 2), we were unable to analyze the complete diversity of bacterial groups present in the maize rhizosphere. A more thorough study, possibly combining culture-independent methods, is therefore necessary to resolve which bacterial communities are associated with the development of SERR symptoms.

The results from our study could be applied to find potential antagonistic bacteria for biocontrol of SERR caused by *Fv*, work which is currently being conducted in our research group. In parallel, we analyzed the bacterial collection described herein by *in vitro* antagonism assays against a pathogenic *Fv* isolate; this yielded 622 bacterial isolates that could inhibit *Fv* growth by 60 to 95%. Furthermore, selection of bacterial antagonists against *Fv* from different *in vitro* assays has enabled identifying *Bacillus* spp. isolates from OTUs

1, 2, 6, 7, 17, 95 and a *Pseudomonas putida* isolate from OTU 36. Isolates belonging to OTUs 2 and 6 (*B. cereus* and *B. megaterium*, respectively) exhibited promising *Fv* antagonistic activity *in planta* using two different white maize hybrids. The research presented here will facilitate the discovery of potential biocontrol agents and bacteria capable of exerting disease control mechanisms against *Fv*, as well as provide important information on the agricultural natural conditions of these bacterial populations in maize plants. In the future, this work should improve the design of biocontrol strategies for fungal soil pathogens.

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Figure captions.

Figure 1. Associated SERR symptoms observed in maize plants from commercial fields. A) SERR symptomatic maize plant (red arrow) exhibiting stem thinning, one of the visible signs of the disease. The yellow arrow points to a healthy-looking asymptomatic maize plant; B) Close-up showing the effect of SERR on stem width and the maize root system. The yellow arrow points to an asymptomatic maize plant with normal stem width and an extensive root system. The red arrow points to a SERR symptomatic maize plant exhibiting a profound decrease in the root system health, accompanied by an underdeveloped stem.

Figure 2. Rarefaction analysis of the 16S rDNA sequences from culturable bacterial isolates from maize rhizospheres. Curves were constructed based on 95 and 97 % sequence identity. Curves did not reach a plateau, indicating that sample saturation was not reached.

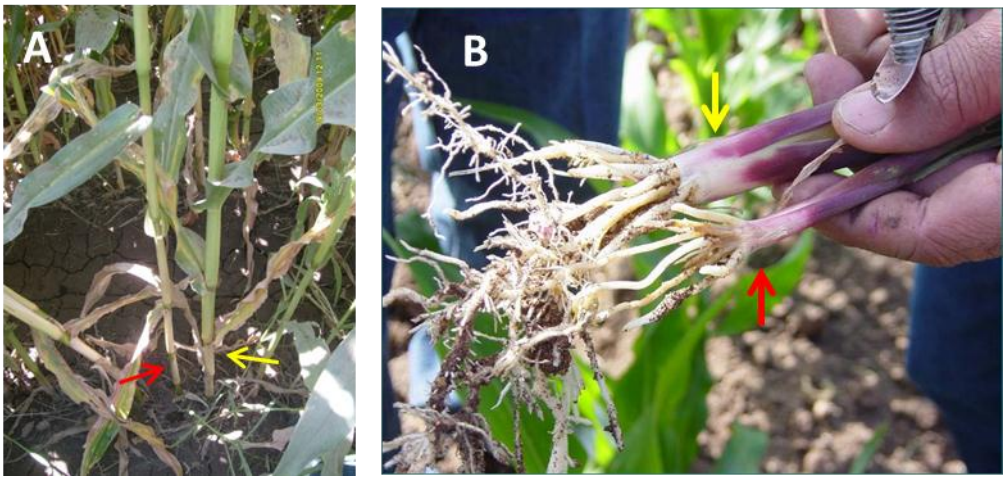
Figure 3. Differential distribution of OTUs for each sampling site. For each Venn diagram, the numbers inside the ovals indicate how many OTUs were present in either asymptomatic (ASY) or SERR symptomatic (SYM) plants, or in both (overlapping areas). Several OTUS were present only in either asymptomatic or symptomatic rhizospheres.

Table captions

Table 1. OTUs specifically associated with either asymptomatic or SERR symptomatic plant rhizospheres.

Table 2. OTUs found in more than one location that showed significant differences (Pr>Chisq >0.05) associated with location and/or infection.

Figure 1.



Identification of culturable bacteria from the rhizosphere of *Fusarium verticillioides* symptomatic and asymptomatic maize plants

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Figure 2.

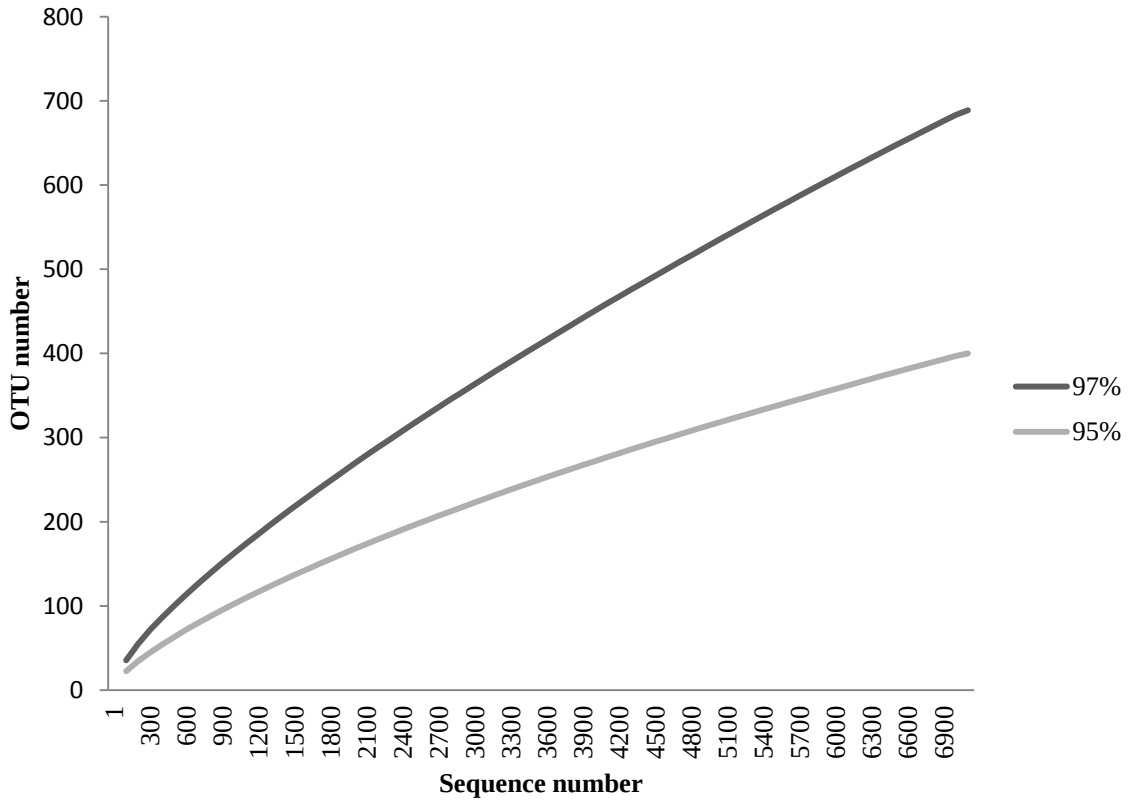

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Figure 3.

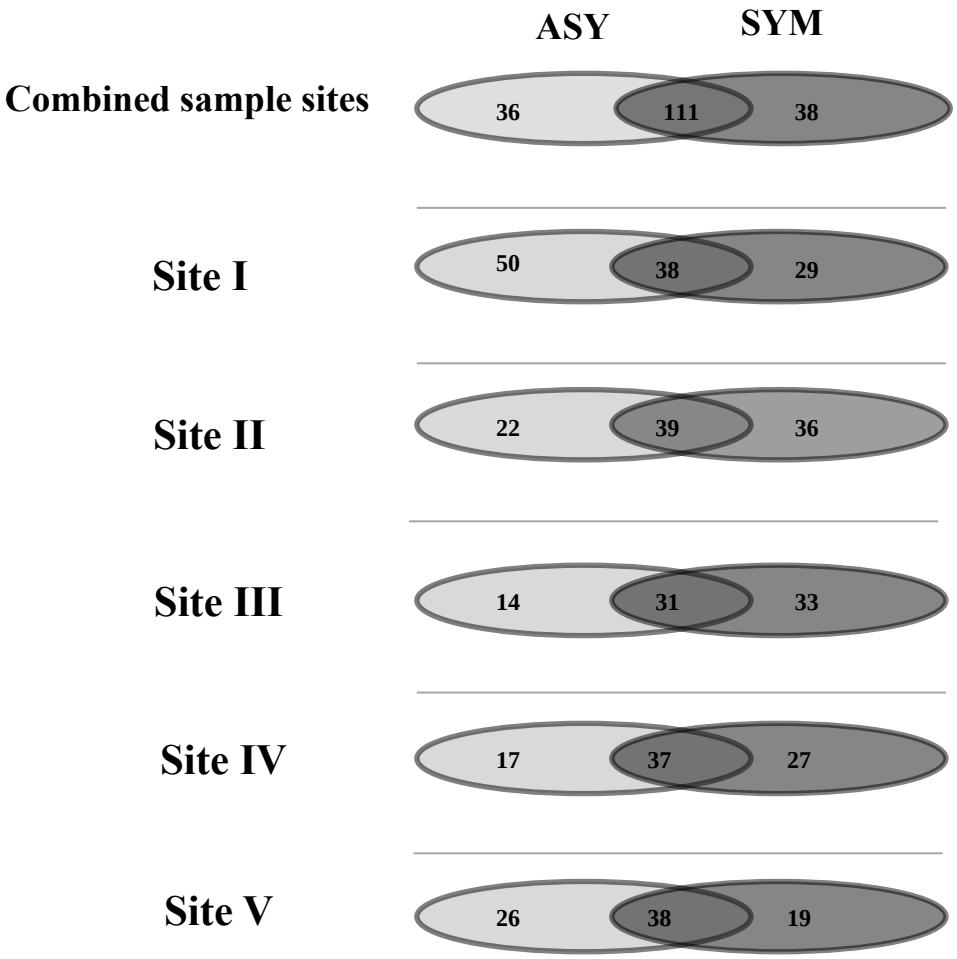

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Table 1. OTUs specifically associated with either asymptomatic or SERR symptomatic plant rhizospheres.

OTUs associated only with asymptomatic plant rhizospheres		
OTU number	Putative species	Sample site
34	B. cereus	I
37	P. putida	I
39	P. putida	I
46	P. putida	I
50	P. putida	I
52	Bacillus sp.	I
54	B. subtilis	I
75	P. putida	I
79	B. subtilis	I
84	B. pumilus	I
313	Enterobacter sp.	I
321	B. pumilus	I
325	B. pumilus	I
331	B. pumilus	I
335	Enterobacter sp.	I
453	Paenibacillus sp.	I
495	B. thuringiensis	I
505	B. megaterium	I
251	B. pumilus	II
600	Pseudomonas sp.	III
607	B. cereus	III
195	B. cereus	IV
167	A. calcoaceticus	V
381	B. thuringiensis	V
151	Bacillus sp.	III / V
162	B. cereus	II / III / V
197	B. catenulatus	I / IV
254	Bacillus sp.	I / II
260	B. cereus	I / II
324	B. pumilus	I / III
336	B. pumilus	I / III
372	B. subtilis	I / V
423	B. aquimaris	II / IV
464	B. subtilis	II / III
494	Bacillus sp.	I / II
510	B. megaterium	I / II

OTUs associated only with symptomatic plant rhizospheres		
OTU number	Putative species	Sample site
104	C. sakazakii	I
577	B. megaterium	I
282	P. mendocina	II
642	B. amyloliquefaciens	III
650	Aeromonas media	III
661	P. fluorescens	III
662	B. megaterium	III
433	B. subtilis	IV
568	B. aquimaris	IV
575	B. aquimaris	IV
392	L. sphaericus	V
393	L. sphaericus	V
409	B. subtilis	V
123	B. subtilis	II / III
124	B. subtilis	I / II
127	B. subtilis	I / II
168	P. putida	II / V
175	Bacillus sp.	I / V
183	B. cereus	I / V
190	B. cereus	I / V
216	Enterobacter sp.	I / II
220	B. subtilis	II / IV
224	B. cereus	II / IV
240	S. maltophila	I / III
243	S. canadense	II / III
274	Brevibacillus sp.	II / IV
281	Paenibacillus sp.	II / III / IV
288	Bacillus sp.	II / IV
289	Rhizobium sp.	II / III
398	Bacillus sp.	I / V
401	B. thuringiensis	IV / V
411	Bacillus sp.	IV / V
441	Terribacillus sp.	I / IV
469	B. thuringiensis	I / III
490	B. subtilis	I / III
592	Bacillus sp.	III / IV
593	B. megaterium	III / IV
523	Bacillus sp.	I / V

Table 2. OTUs found in more than one location that showed significant differences (Pr>Chisq >0.05) associated with location and/or infection.

OTU	Species	Pr > Chisq		OTU site presence	Infection status
		Location	Infection		
1	*B. subtilis*	0.0001	0.0031	all five	SYM
2	*B. cereus*	0.0001	0.0001	all five	SYM
5	*B. megaterium*	0.0001		all five	
6	*Bacillus* sp.	0.0001		all five	
7	*B. cereus*	0.0001	0.0002	all five	SYM
8	*B. subtilis*	0.0001		all five	
12	*B. subtilis*	0.0001		all five	
13	*B. endophyticus*	0.0001		all five	
17	*B. thuringiensis*	0.0001	0.02	all five	ASY
18	*B. subtilus*	0.0001		all five	
19	*B. firmus*	0.0001		all five	
21	*B. megaterium*	0.0001		all five	
22	*E. cloacae*	0.0001	0.001	I, II, III	SYM
36	*P.putida*	0.0049	0.0038	all five	ASY
82	*Pantoea* sp.	0.0001	0.0001	I, V	SYM
94	*L. sphaericus*	0.0001	0.0001	all five	SYM
95	*B. megaterium*	0.0001		all five	
114	*Arthrobacter* sp.	0.0001		II, IV, V	
117	*B. niacini*	0.0001		all five	
128	*B. megaterium*	0.0001		I, II, IV, V	
169	*Acinetobacter* sp.	0.0016		I, V	
179	*B. luciferensis*	0.0439		all five	
239	*S. maltophila*	0.0015		I, II, III	
375	*L. sphaericus*	0.0039		I, V	
383	*P. fluorescens*	0.0001	0.0001	III, IV, V	ASY
388	*B. aquimaris*	0.027		III, IV, V	
422	*Paenibacillus* sp.	0.0237		I, II, III, IV	

Table S1. Physicochemical properties of sampled soils

SITE / INFECTION	Sample number	pH (1:2)	Electric Conductivity (EC)	Organic matter (%)	Total nitrogen (ppm)	Bioavailable P (mg/Kg)	K (Cmol/Kg)	Ca (Meq/100g)	Mg (Cmol/Kg)	Na (Cmol/Kg)	TEXTURE			TYPE OF TEXTURE	COMPANY/MAIZE HYBRID	Days after sowing	Shannon Diversity Index
											SAND (%)	SILT (%)	CLAY (%)				
	1	6.7	0.01	0.26	51	12.96	1.11	7.48	2.95	1.24							
	2	6.7	0.06	0.40	52	12.68	0.81	8.31	1.94	1.24							
I / SYM	3	6.7	0.03	0.40	41	21.03	1.07	7.89	2.82	1.24							2.65
	4	6.7	0.04	0.26	51	10.95	0.98	8.31	3.30	2.40							
	5	6.3	0.02	0.40	52	5.18	0.81	7.06	3.07	1.24							
	Average	6.6 (*)	0.03	0.34	49.4	12.56	0.95	7.81	2.81	1.47	57.06	32.70	10.24	Sandy-loam	PIONEER / 30P49	40-45	
	6	6.2	0.04	0.67	45	12.10	1.57	10.59	3.09	1.24							
	7	5.7	0.02	0.40	42	10.66	0.90	11.22	3.11	1.24							
I / ASYM	8	5.7	0.02	0.40	42	10.66	0.90	11.22	3.11	1.24							3.29
	9	6.5	0.03	0.13	48	6.62	0.81	7.48	2.08	1.24							
	10	6.0	0.03	0.26	50	12.39	0.90	6.70	3.42	1.24							
	Average	6.02 (*)	0.02	0.37	45.4	10.48	1.01	9.44	2.96	1.24							
	11	7.9	0.09	0.26	60	12.10	1.26	13.71	3.67	2.40							
	12	8.0	0.10	0.67	49	13.83	1.46	7.68	4.07	2.40							
II / SYM	13	7.7	0.06	0.40	48	13.25	1.54	11.01	2.33	2.40							2.52
	14	7.7	0.06	0.26	58	16.13	1.54	12.26	4.60	1.24							
	15	7.9	0.08	0.40	49	17.86	1.63	7.86	4.59	1.24							
	Average	7.8 (*)	0.07	0.39	52.8	14.63	1.48	10.50	3.85	1.93	51.6	54.70	14.24	Clay	DEKALB / DK2020	80-90	
	16	7.5	0.07	0.53	49	19.59	1.37	16.83	3.13	1.24							
	17	7.7	0.06	0.13	56	16.13	1.07	11.63	1.58	1.24							
II / SYM	18	7.4	0.06	0.26	55	32.56	1.37	7.06	2.35	1.24							2.49
	19	7.4	0.19	0.67	44	27.37	1.80	13.30	3.30	2.40							
	20	7.4	0.07	0.26	58	14.69	1.90	11.01	3.64	1.24							
	Average	7.4 (*)	0.09	0.37	52.4	22.06	1.50	11.96	2.80	1.47							
	21	7.8	0.03	0.40	55	21.03	1.07	7.89	2.82	1.24							
	22	7.7	0.10	0.40	55	19.02	1.67	8.10	3.65	1.24							
III / SYM	23	7.1	0.23	0.53	55	14.69	1.54	12.05	3.53	2.40							2.75
	24	7.7	0.08	0.40	69	15.85	1.50	9.56	3.31	2.40							
	25	7.5	0.15	0.26	66	12.96	1.33	11.63	3.37	2.40							
	Average	7.5	0.11	0.39	60	16.71	1.42	9.84	3.33	1.93	27.06	56.70	10.24	Clay	SYNGENTA / RENACER	60-70	
	26	7.2	0.12	0.53	48	38.60	1.20	13.71	4.94	2.40							
	27	7.2	0.09	0.40	63	20.46	1.33	11.34	2.76	1.24							
III / ASYM	28	7.5	0.09	0.26	46	11.23	1.46	13.71	3.31	1.24							2.44
	29	7.6	0.08	0.53	42	11.23	1.59	8.31	6.30	2.40							
	30	7.4	0.07	0.40	49	13.83	1.30	11.60	4.46	1.24							
	Average	7.3	0.09	0.42	49.6	19.07	1.37	11.73	4.35	1.70							
	31	7.2	0.08	0.53	59	42.93	2.06	19.32	5.76	1.24							
	32	7.1	0.10	0.67	59	44.09	2.02	19.31	6.17	1.24							
IV / SYM	33	7.1	0.07	0.26	49	38.04	1.76	17.56	5.76	2.48							2.39
	34	7.2	0.08	0.40	55	63.11	1.33	18.28	5.76	1.24							
	35	7.2	0.08	0.40	59	31.98	1.41	17.66	5.34	1.24							
	Average	7.1	0.08	0.45	56.2	44.03	1.71	18.42	5.75	1.48	8.34	40.70	50.96	Silty-clay	PIONEER / 30P49	70-80	
	36	6.9	0.19	0.26	49	42.36	1.71	15.79	5.76	1.24							
	37	6.9	0.10	0.67	48	26.80	1.41	13.30	5.76	1.24							
IV / ASYM	38	6.8	0.11	0.67	48	36.59	1.59	18.28	5.76	1.24							2.27
	39	7.0	0.12	0.26	56	34.00	1.67	14.96	5.76	1.24							
	40	7.6	0.06	0.93	60	18.15	1.11	6.61	5.34	2.48							
	Average	7.0	0.11	0.55	52.2	31.58	1.49	13.78	5.67	1.48							
	41	7.6	0.06	0.93	60	18.15	1.11	6.61	5.34	2.48							
	42	7.4	0.13	0.26	52	5.18	1.07	6.02	5.76	2.48							
V / SYM	43	7.2	0.13	0.26	48	5.18	1.16	5.05	5.76	1.24							2.2
	44	7.8	0.05	0.40	53	4.32	0.77	23.06	5.34	2.48							
	45	7.5	0.06	0.40	55	6.05	0.98	7.68	5.76	2.48	18.34	28.70	52.96	Clay	DEKALB / DK2020	120-130	
	Average	7.5	0.08	0.45	53.6	7.77	1.01	9.68	5.59	2.23							
	46	7.6	0.15	0.26	56	8.06	1.16	16.52	5.34	2.48							
V / ASYM	47	5.4	0.13	0.26	55	7.20	1.07	10.55	5.76	1.24							2.29

	48	7.5	0.13	0.40	56	9.22	1.16	8.05	4.93	1.24							
	49	7.4	0.05	0.80	56	8.64	1.28	10.39	5.76	1.24							
	50	7.7	0.07	0.67	60	14.69	1.16	9.06	4.52	1.24							
	Average	7.1	0.10	0.47	56.6	9.56	1.16	10.91	5.26	1.48							
One way ANOVA P> 0.01 No differences were found by contrasting values in each site of SERR symptomatic versus asymptomatic soil samples.																	
(*) comparison shows statistical difference between samples from both conditions in this site for this variable.																	
Soil texture was analyzed per site since SERR symptomatic and asymptomatic plants were adjacent to each other.																	

Table S2. Relative abundance and diversity of phyla from culturable bacteria found in the maize rhizosphere of SERR symptomatic and asymptomatic plants.

Phyla	Genus	Number of OTUs	Number of sequences (% of total)
Firmicutes	*Bacillus*	131	5332 (81.12%)
	Lysinibacillus	5	282 (4.29%)
	Terribacillus	2	17 (0.26%)
	Paenibacillus	6	45 (0.69%)
	Staphylococcus	1	8 (0.12%)
	Brevibacillus	1	3 (0.04%)
	6	**146**	**5, 687 (86.52%)**
Proteobacteria (class)			
Alphaproteobacteria	*Rhizobium*	1	4 (0.06%)
Betaproteobacteria	*Massilia*	1	2 (0.03%)
Gammaproteobacteria	*Pseudomonas*	15	252 (3.83%)
	Enterobacter	5	407 (6.22%)
	Klebsiella	3	10 (0.15%)
	Pantoea	2	71 (1.08%)
	Cronobacter	1	2 (0.03%)
	Acinetobacter	4	35 (0.53%)
	Stenotrophomonas	2	42 (0.64%)
	Aeromonas	1	18 (0.28%)
	10	**34**	**843 (12.81%)**
Actinobacteria	*Arthrobacter*	2	31 (0.47%)
	Sinomonas	1	4 (0.06%)
	2	**3**	**35 (0.53%)**
Bacteroidetes	*Sphingobacterium*	1	6 (0.11%)
	1	**1**	**6 (0.11%)**
Not determined	Not determined	1	2 (0.03%)
	1	**1**	**2 (0.03%)**
TOTAL	**19**	**185**	**5,673 (100%)**

Table S3. Frequency and abundance of putative species assigned by BLAST-N and phylogenetic analysis.

Phyla	Genera	Putative species	OTU frequency	Number of sequences
Actinobacteria	*Arthrobacter*	*Arthrobacter* sp.	1	28
		A. nitroguajacolicus	1	3
	Sinomonas	*S. atrocyanea*	1	4
Bacteroidetes	*Sphingobacterium*	*S. canadense*	1	6
Firmicutes	*Bacillus*	*B. amyloliquefaciens*	1	2
		B. anthracis	1	2
		B. aquimaris	4	37
		B. catenulatus	1	3
		B. cereus	19	1402
		B. circulans	1	8
		B. endophyticus	2	74
		B. firmus	1	152
		B. flexus	3	10
		B. luciferensis	1	8
		B. megaterium	15	808
		B. niacini	1	91
		B. pumilus	10	35
		B. subtilis	28	912
		B. thuringiensis	7	92
		Bacillus sp.	36	1696
	Brevibacillus	*Brevibacillus* sp.	1	3
	Lysinibacillus	*L. sphaericus*	5	282
	Paenibacillus	*P. polymyxa*	1	12
		Paenibacillus sp.	5	33
	Staphylococcus	*Staphylococcus* sp.	1	8
	Terribacillus	*Terribacillus* sp.	2	17
Proteobacteria	α-Proteobacteria/*Rhizobium*	*Rhizobium* sp.	1	4
	β-Proteobacteria/*Massilia*	*M. timonae*	1	2
	γ-Proteobacteria/*Acinetobacter*	*A. calcoaceticus*	3	18
		Acinetobacter sp.	1	17
	Aeromonas	*A. media*	1	18
	Cronobacter	*C. sakazakii*	1	2
	Enterobacter	*E. cloacae*	1	399
		Enterobacter sp.	3	8
	Klebsiella	*K. pneumoniae*	1	4
		Klebsiella sp.	2	6
	Pseudomonas	*P. fluorescens*	3	139

		P. luteola	1	4
		P. mendocina	1	2
		P. putida	8	102
		Pseudomonas sp.	2	5
	Pantoea	*P. agglomerans*	1	2
		Pantoea sp.	1	69
	Stenotrophomonas	*S. maltophila*	2	42
not determined	not determined	not determined	1	2
		TOTAL	185	6573

NOTE: Names highlighted in blue indicate the nine most abundant species

Table S4. The abundance and distribution of OTUs in different locations, as associated with SERR symptomatic (SYM) and asymptomatic (ASY) maize rhizospheres, measured as total abundance (total sequences) and relative abundance (percentage of the total).

Column number			Putative Species	Site I		Site II		Site III		Site IV		Site V		Total sequences	Percentage of the total
	OTU Number	Phylum or Class/Genus		ASY	SYM	ASY	SYM	ASY	SYM	ASY	SYM	ASY	SYM		
1	**OTU 1**	Firmicutes/*Bacillus*	*B. subtilis*	86	41	59	141	43	79	64	57	16	23	609	**8.6**
2	**OTU 2**	Firmicutes/*Bacillus*	*B. cereus*	60	57	55	117	45	85	96	98	167	205	985	**13.9**
3	**OTU 3**	Firmicutes/*Bacillus*	*Bacillus* sp.	1			1							2	**0.02**
4	**OTU 4**	Firmicutes/*Bacillus*	*B. cereus*	7	5	8	14	5	8	12	14	10	13	96	**1.3**
5	**OTU 5**	Firmicutes/*Bacillus*	*B. megaterium*	34	33	20	13	20	13	17	17	9	15	191	**2.6**
6	**OTU 6**	Firmicutes/*Bacillus*	*Bacillus* sp.	186	239	174	107	123	181	198	172	82	94	1556	**21.9**
7	**OTU 7**	Firmicutes/*Bacillus*	*B. cereus*	12	5	26	39	9	17	14	29	31	49	231	**3.2**
8	**OTU 8**	Firmicutes/*Bacillus*	*B. subtilis*	6	8	6	9	2	5	2	3	2	1	44	**0.6**
9	**OTU 10**	Firmicutes/*Staphylococcus*	*Staphylococcus* sp.		1		4			2		1		8	**0.11**
10	**OTU 12**	Firmicutes/*Bacillus*	*B. subtilis*	8	11	12	23	2	3	13	6	5	3	86	**1.2**
11	**OTU 13**	Firmicutes/*Bacillus*	*B. endophyticus*	2		15	15	9	10	2	1	8	8	70	**0.9**
12	**OTU 15**	Firmicutes/*Bacillus*	*B. cereus*	2		2	3	2	1	4	4	2	5	25	**0.35**
13	**OTU 16**	Firmicutes/*Bacillus*	*B. subtilis*	4	3	1	3		3	3		3		20	**0.28**
14	**OTU 17**	Firmicutes/*Bacillus*	*B. thuringiensis*	7	2	7	26	4		6	12	3	7	74	**1.04**
15	**OTU 18**	Firmicutes/*Bacillus*	*B. subtilis*	5	6	5	18	1	1	8	3	6		53	**0.7**
16	**OTU 19**	Firmicutes/*Bacillus*	*B. firmus*	6	4	10	15	25	41	18	3	12	18	152	**2.1**
17	**OTU 20**	Firmicutes/*Bacillus*	*B. flexus*				1			1	1	1		4	**0.05**
18	**OTU 21**	Firmicutes/*Bacillus*	*B. megaterium*	32	47	25	22	15	15	31	17	9	12	225	**3.1**
19	**OTU 22**	γ-Proteobacteria/*Enterobacter*	*E. cloacae*	132	208	2	18	1	38					399	**5.6**
20	**OTU 23**	Firmicutes/*Bacillus*	*B. megaterium*	4		1	2		1		1			9	**0.1**
21	**OTU 25**	Firmicutes/*Bacillus*	*Bacillus* sp.				2					3	2	7	**0.9**
22	**OTU 26**	Firmicutes/*Bacillus*	*B. cereus*				1			2	1	1	5	10	**0.1**
23	**OTU 30**	Firmicutes/*Terribacillus*	*Terribacillus* sp.	3	1	4	2		3		2			15	**0.2**

24	**OTU 34**	Firmicutes/*Bacillus*	*B. cereus*	2										2	**0.02**
25	**OTU 36**	γ-Proteobacteria/*Pseudomonas*	*P. putida*	21	3	13	7		14	22	2	1	4	87	**1.2**
26	**OTU 37**	γ-Proteobacteria/*Pseudomonas*	*P. putida*	3										3	**0.04**
27	**OTU 39**	γ-Proteobacteria/*Pseudomonas*	*P. putida*	2										2	**0.02**
28	**OTU 43**	Firmicutes/*Bacillus*	*B. subtilis*	3	1	1				2	3	1	1	12	**0.1**
29	**OTU 46**	γ-Proteobacteria/*Pseudomonas*	*P. putida*	2										2	**0.02**
30	**OTU 50**	γ-Proteobacteria/*Pseudomonas*	*P. putida*	2										2	**0.02**
31	**OTU 51**	Firmicutes/*Bacillus*	*B. thuringiensis*	2					1					3	**0.04**
32	**OTU 52**	Firmicutes/*Bacillus*	*Bacillus* sp.	2										2	**0.02**
33	**OTU 54**	*Firmicutes/Bacillus*	*B. subtilis*	3										3	**0.04**
34	**OTU 55**	γ-Proteobacteria/*Klebsiella*	*K. pneumoniae*	3	1									4	**0.05**
35	**OTU 56**	γ-Proteobacteria/*Pseudomonas*	*P. putida*	1			1							2	**0.02**
36	**OTU 59**	γ-Proteobacteria/*Klebsiella*	*Klebsiella sp.*	1	1									2	**0.02**
37	**OTU 60**	Firmicutes/*Bacillus*	*B. subtilis*	1					1			1		3	**0.04**
38	**OTU 75**	γ-Proteobacteria/*Pseudomonas*	*P. putida*	2										2	**0.02**
39	**OTU 79**	Firmicutes/*Bacillus*	*B. subtilis*	2										2	**0.02**
40	**OTU 82**	γ-Proteobacteria/*Pantoea*	*Pantoea sp.*	2	66							1		69	**0.97**
41	**OTU 84**	Firmicutes/*Bacillus*	*B. pumilus*	2										2	**0.02**
42	**OTU 94**	Firmicutes/*Lysinibacillus*	*L. sphaericus*	3	26	3	1	2	12	24	32	29	128	260	**3.67**
43	**OTU 95**	Firmicutes/*Bacillus*	*B. megaterium*	37	28	41	35	3	15	37	16	11	8	231	**3.26**
44	**OTU 98**	Firmicutes/*Bacillus*	*Bacillus* sp.		1	1		1	1	2	1	2		9	**0.12**
45	**OTU 103**	Firmicutes/*Paenibacillus*	*P. polymyxa*		1	1	1	3	2		1	2	1	12	**0.16**
46	**OTU 104**	γ-Proteobacteria/*Cronobacter*	*C. sakazakii*		2									2	**0.02**
47	**OTU 112**	β-Proteobacteria/*Massilia*	*M. timonae*									1	1	2	**0.02**
48	**OTU 113**	Firmicutes/*Bacillus*	*Bacillus* sp.		1	1	2	1	2	2		9	1	19	**0.26**
49	**OTU 114**	Actinobacteria/*Arthrobacter*	*Arthrobacter sp.*			10	13			1	2	1	1	28	**0.39**
50	**OTU 117**	Firmicutes/*Bacillus*	*B. niacini*	27	14	2	9	5	6	11	4	9	4	91	**1.28**
51	**OTU 118**	Firmicutes/*Bacillus*	*Bacillus* sp.		2							1	1	4	**0.05**

52	**OTU 120**	γ-Proteobacteria/*Acinetobacter*	*A. calcoaceticus*		1			1	4			8		14	**0.19**
53	**OTU 121**	Firmicutes/*Bacillus*	*Bacillus* sp.			1						1	2	4	**0.05**
54	**OTU 123**	Firmicutes/*Bacillus*	*B. subtilis*				2		1					3	**0.04**
55	**OTU 124**	Firmicutes/*Bacillus*	*B. subtilis*		1		1							2	**0.02**
56	**OTU 125**	Firmicutes/*Bacillus*	*B. subtilis*	1	1		3		1					6	**0.08**
57	**OTU 126**	Firmicutes/*Bacillus*	*B. circulans*	1		1	2		2	1		1		8	**0.11**
58	**OTU 127**	Firmicutes/*Bacillus*	*B. subtilis*		1		1							2	**0.02**
59	**OTU 128**	Firmicutes/*Bacillus*	*B. megaterium*	10	15	7	5			5	9	1		52	**0.73**
60	**OTU 129**	Firmicutes/*Bacillus*	*B. endophyticus*			3	1							4	**0.05**
61	**OTU 130**	Firmicutes/*Bacillus*	*B. megaterium*	6	1	3	4	1	4	1	4	2	1	27	**0.38**
62	**OTU 131**	Firmicutes/*Bacillus*	*B. pumilus*	2	4		3		2		1	4		16	**0.22**
63	**OTU 134**	Firmicutes/*Bacillus*	*B. subtilis*		4	4	4	2		5		2		21	**0.29**
64	**OTU 137**	Firmicutes/*Bacillus*	*B. subtilis*		2		2		1	2	2			9	**0.12**
65	**OTU 138**	Firmicutes/*Bacillus*	*B. subtilis*			1	1							2	**0.02**
66	**OTU 141**	Firmicutes/*Bacillus*	*B. pumilus*			1	1							2	**0.02**
67	**OTU 151**	Firmicutes/*Bacillus*	*Bacillus* sp.					1				1		2	**0.02**
68	**OTU 156**	Firmicutes/*Bacillus*	*B. subtilis*		1		1		1			1		4	**0.05**
69	**OTU 157**	Firmicutes/*Bacillus*	*Bacillus* sp.						2	1		1		4	**0.05**
70	**OTU 159**	Firmicutes/*Bacillus*	*Bacillus* sp.	2			2	2	3	2	3	2	2	18	**0.25**
71	**OTU 162**	Firmicutes/*Bacillus*	*B. cereus*			1		2				3		6	**0.08**
72	**OTU 163**	Firmicutes/*Bacillus*	*Bacillus* sp.									1	1	2	**0.02**
73	**OTU 164**	γ-Proteobacteria/*Acinetobacter*	*A. calcoaceticus*									1	1	2	**0.02**
74	**OTU 167**	γ-Proteobacteria/*Acinetobacter*	*A. calcoaceticus*									2		2	**0.02**
75	**OTU 168**	γ-Proteobacteria/*Pseudomonas*	*P. putida*				1						1	2	**0.02**
76	**OTU 169**	γ-Proteobacteria/*Acinetobacter*	*Acinetobacter* sp.	8	7								2	17	**0.24**
77	**OTU 172**	Firmicutes/*Bacillus*	*B. anthracis*			1							1	2	**0.02**
78	**OTU 174**	Actinobacteria/*Sinomonas*	*S. atrocyanea*				2			1			1	4	**0.05**
79	**OTU 175**	Firmicutes/*Bacillus*	*Bacillus* sp.		1								1	2	**0.02**

80	**OTU 179**	Firmicutes/*Bacillus*	*B. luciferensis*	1						4	2		1	8	**0.11**
81	**OTU 183**	Firmicutes/*Bacillus*	*B. cereus*		1								1	2	**0.02**
82	**OTU 185**	γ-Proteobacteria/*Pseudomonas*	*P. luteola*									2	2	4	**0.05**
83	**OTU 190**	Firmicutes/*Bacillus*	*B. cereus*		1								1	2	**0.02**
84	**OTU 195**	Firmicutes/*Bacillus*	*B. cereus*							2				2	**0.02**
85	**OTU 196**	Firmicutes/*Bacillus*	*B. cereus*		1					2	1			4	**0.05**
86	**OTU 197**	Firmicutes/*Bacillus*	*B. catenulatus*	1						2				3	**0.04**
87	**OTU 202**	Firmicutes/*Bacillus*	*Bacillus* sp.							1	1			2	**0.02**
88	**OTU 208**	Firmicutes/*Bacillus*	*Bacillus* sp.	1			1							2	**0.02**
89	**OTU 212**	Firmicutes/*Bacillus*	*B. cereus*	1			1			2	1			5	**0.07**
90	**OTU 216**	γ-Proteobacteria/*Enterobacter*	*Enterobacter* sp.		2		1							3	**0.04**
91	**OTU 220**	Firmicutes/*Bacillus*	*B. subtilis*				1				1			2	**0.02**
92	**OTU 221**	N/D	N/D	1			1							2	**0.02**
93	**OTU 224**	Firmicutes/*Bacillus*	*B. cereus*				1				1			2	**0.02**
94	**OTU 228**	Firmicutes/*Bacillus*	*B. cereus*					1					1	2	**0.02**
95	**OTU 231**	Firmicutes/*Bacillus*	*B. subtilis*	1		1	2	1						5	**0.07**
96	**OTU 239**	γ-Proteobacteria/*Stenotrophomonas*	*S. maltophila*		3	7	14	9	7					40	**0.56**
97	**OTU 240**	γ-Proteobacteria/*Stenotrophomonas*	*S. maltophila*		1				1					2	**0.02**
98	**OTU 243**	Bacteroidetes/*Sphingobacterium*	*S. canadense*				1		5					6	**0.08**
99	**OTU 248**	Actinobacteria/*Arthrobacter*	*A. nitroguajacolicus*		1	2								3	**0.04**
100	**OTU 251**	Firmicutes/*Bacillus*	*B. pumilus*			2								2	**0.02**
101	**OTU 252**	Firmicutes/*Bacillus*	*Bacillus sp.*			1					1			2	**0.02**
102	**OTU 253**	Firmicutes/*Bacillus*	*B. cereus*			1	1	1				1	1	5	**0.07**
103	**OTU 254**	Firmicutes/*Bacillus*	*Bacillus sp.*	1		1								2	**0.02**
104	**OTU 259**	Firmicutes/*Bacillus*	*B. pumilus*	1		1	1							3	**0.04**
105	**OTU 260**	Firmicutes/*Bacillus*	*B. cereus*	2		1								3	**0.04**
106	**OTU 261**	Firmicutes/*Bacillus*	*B. flexus*	1			1							2	**0.02**
107	**OTU 262**	Firmicutes/*Bacillus*	*Bacillus* sp.	1			1							2	**0.02**

108	**OTU 266**	Firmicutes/*Bacillus*	*B. thuringiensis*	1	1		2	1			1		1	7	**0.09**
109	**OTU 267**	Firmicutes/*Bacillus*	*B. megaterium*	3	8	4	1			4	7	6	2	35	**0.49**
110	**OTU 268**	Firmicutes/*Bacillus*	*B. subtilis*				1			1				2	**0.02**
111	**OTU 269**	Firmicutes/*Bacillus*	*Bacillus* sp.	1	2		1							4	**0.05**
112	**OTU 270**	Firmicutes/*Bacillus*	*B. megaterium*	7	1	1	2	1	1	4	3	1	2	23	**0.32**
113	**OTU 272**	Firmicutes/*Bacillus*	*B. cereus*				2				1	1		4	**0.05**
114	**OTU 273**	Firmicutes/*Bacillus*	*B. cereus*	1		1	4	3	2				3	14	**0.19**
115	**OTU 274**	Firmicutes/*Brevibacillus*	*Brevibacillus* sp.				1				2			3	**0.04**
116	**OTU 275**	Firmicutes/*Bacillus*	*B. subtilis*	1			1							2	**0.02**
117	**OTU 281**	Firmicutes/*Paenibacillus*	*Paenibacillus* sp.				2		1		1			4	**0.05**
118	**OTU 282**	γ-Proteobacteria/*Pseudomonas*	*P. mendocina*				2							2	**0.02**
119	**OTU 288**	Firmicutes/*Bacillus*	*Bacillus* sp.				1				1			2	**0.02**
120	**OTU 289**	α-Proteobacteria/*Rhizobium*	*Rhizobium* sp.				3		1					4	**0.05**
121	**OTU 313**	γ-Proteobacteria/*Enterobacter*	*Enterobacter* sp.	3										3	**0.04**
122	**OTU 314**	γ-Proteobacteria/*Pantoea*	*Pantoea agglomerans*	1	1									2	**0.02**
123	**OTU 321**	Firmicutes/*Bacillus*	*B. pumilus*	2										2	**0.02**
124	**OTU 324**	Firmicutes/*Bacillus*	*B. pumilus*	1				1						2	**0.02**
125	**OTU 325**	Firmicutes/*Bacillus*	*B. pumilus*	2										2	**0.02**
126	**OTU 331**	Firmicutes/*Bacillus*	*B. pumilus*	2										2	**0.02**
127	**OTU 335**	γ-Proteobacteria/*Enterobacter*	*Enterobacter* sp.	2										2	**0.02**
128	**OTU 336**	Firmicutes/*Bacillus*	*B. pumilus*	1				1						2	**0.02**
129	**OTU 365**	Firmicutes/*Bacillus*	*B. megaterium*	1	1									2	**0.02**
130	**OTU 368**	γ-Proteobacteria/*Klebsiella*	*Klebsiella* sp.	2	2									4	**0.05**
131	**OTU 372**	Firmicutes/*Bacillus*	*B. subtilis*	1								1		2	**0.02**
132	**OTU 375**	Firmicutes/*Lysinibacillus*	*L. sphaericus*	1								8	3	12	**0.16**
133	**OTU 381**	Firmicutes/*Bacillus*	*B. thuringiensis*									2		2	**0.02**
134	**OTU 383**	γ-Proteobacteria/*Pseudomonas*	*P. fluorescens*					109	12	5		5	4	135	**1.9**
135	**OTU 385**	Firmicutes/*Lysinibacillus*	*L. sphaericus*	1							3	2		6	**0.08**

136	**OTU 387**	Firmicutes/*Paenibacillus*	*Paenibacillus* sp.							1	2	1		4	**0.05**
137	**OTU 388**	Firmicutes/*Bacillus*	*B. aquimaris*					4	5	11	6	1	4	31	**0.43**
138	**OTU 390**	Firmicutes/*Bacillus*	*Bacillus* sp.								1	1		2	**0.02**
139	**OTU 392**	Firmicutes/*Lysinibacillus*	*L. sphaericus*										2	2	**0.02**
140	**OTU 393**	Firmicutes/*Lysinibacillus*	*L. sphaericus*										2	2	**0.02**
141	**OTU 394**	Firmicutes/*Paenibacillus*	*Paenibacillus* sp.		1	3					4		2	10	**0.14**
142	**OTU 398**	Firmicutes/*Bacillus*	*Bacillus* sp.		1								1	2	**0.02**
143	**OTU 401**	Firmicutes/*Bacillus*	*B. thuringiensis*								1		1	2	**0.02**
144	**OTU 409**	Firmicutes/*Bacillus*	*B. subtilis*										3	3	**0.04**
145	**OTU 411**	Firmicutes/*Bacillus*	*Bacillus* sp.								1		1	2	**0.02**
146	**OTU 412**	Firmicutes/*Bacillus*	*B. flexus*							2	2			4	**0.05**
147	**OTU 416**	Firmicutes/*Bacillus*	*Bacillus* sp.						1	1	1			3	**0.04**
148	**OTU 419**	γ-Proteobacteria/*Pseudomonas*	*P. fluorescens*							1			1	2	**0.02**
149	**OTU 421**	γ-Proteobacteria/*Pseudomonas*	*Pseudomonas* sp.						1	1				2	**0.02**
150	**OTU 422**	Firmicutes/*Paenibacillus*	*Paenibacillus* sp.	1	1		1	6	2	2				13	**0.18**
151	**OTU 423**	Firmicutes/*Bacillus*	*B. aquimaris*			1				1				2	**0.02**
152	**OTU 433**	Firmicutes/*Bacillus*	*B. subtilis*								2			2	**0.02**
153	**OTU 441**	Firmicutes/*Terribacillus*	*Terribacillus* sp.		1						1			2	**0.02**
154	**OTU 451**	Firmicutes/*Bacillus*	*Bacillus* sp.	2	1							1	1	5	**0.07**
155	**OTU 453**	Firmicutes/*Paenibacillus*	*Paenibacillus sp.*	2										2	**0.02**
156	**OTU 454**	Firmicutes/*Bacillus*	*Bacillus* sp.	1		1			2					4	**0.05**
157	**OTU 456**	Firmicutes/*Bacillus*	*Bacillus* sp.	2							1	1		4	**0.05**
158	**OTU 459**	Firmicutes/*Bacillus*	*B. subtilis*	2		1		2			1			6	**0.08**
159	**OTU 464**	Firmicutes/*Bacillus*	*B. subtilis*			1		1						2	**0.02**
160	**OTU 469**	Firmicutes/*Bacillus*	*B. thuringiensis*		1				1					2	**0.02**
161	**OTU 489**	Firmicutes/*Bacillus*	*Bacillus* sp.			1			1					2	**0.02**
162	**OTU 490**	Firmicutes/*Bacillus*	*B. subtilis*		1				1					2	**0.02**
163	**OTU 494**	Firmicutes/*Bacillus*	*Bacillus* sp.	1		1								2	**0.02**

164	**OTU 495**	Firmicutes/*Bacillus*	*B. thuringiensis*	2										2	**0.02**
165	**OTU 505**	Firmicutes/*Bacillus*	*B. megaterium*	2										2	**0.02**
166	**OTU 510**	Firmicutes/*Bacillus*	*B. megaterium*	1		1								2	**0.02**
167	**OTU 521**	Firmicutes/*Bacillus*	*Bacillus* sp.		1				1	2		1		5	**0.07**
168	**OTU 523**	Firmicutes/*Bacillus*	*Bacillus* sp.		4				1					5	**0.07**
169	**OTU 541**	Firmicutes/*Bacillus*	*B. subtilis*						1			2		3	**0.04**
170	**OTU 567**	Firmicutes/*Bacillus*	*Bacillus* sp.		1			1	1	1				4	**0.05**
171	**OTU 568**	Firmicutes/*Bacillus*	*B. aquimaris*								2			2	**0.02**
172	**OTU 575**	Firmicutes/*Bacillus*	*B. aquimaris*								2			2	**0.02**
173	**OTU 576**	Firmicutes/*Bacillus*	*Bacillus* sp.			1					1			2	**0.02**
174	**OTU 577**	Firmicutes/*Bacillus*	*B. megaterium*		2									2	**0.02**
175	**OTU 584**	Firmicutes/*Bacillus*	*Bacillus* sp.		1	1								2	**0.02**
176	**OTU 592**	Firmicutes/*Bacillus*	*Bacillus* sp.						1		1			2	**0.02**
177	**OTU 593**	Firmicutes/*Bacillus*	*B. megaterium*						1		1			2	**0.02**
178	**OTU 596**	Firmicutes/*Bacillus*	*B. megaterium*			1			1		1			3	**0.04**
179	**OTU 600**	γ-Proteobacteria/*Pseudomonas*	*Pseudomonas* sp.					3						3	**0.04**
180	**OTU 607**	Firmicutes/*Bacillus*	*B. cereus*					2						2	**0.02**
181	**OTU 625**	Firmicutes/*Bacillus*	*Bacillus* sp.			1		1	1					3	**0.04**
182	**OTU 642**	Firmicutes/*Bacillus*	*B. amyloliquefaciens*						2					2	**0.02**
183	**OTU 650**	γ-Proteobacteria/*Aeromonas*	*A. media*						18					18	**0.25**
184	**OTU 661**	γ-Proteobacteria/*Pseudomonas*	*P. fluorescens*						2					2	**0.02**
185	**OTU 662**	Firmicutes/*Bacillus*	*B. megaterium*						2					2	**0.02**
Total sequences				**812**	**899**	**565**	**752**	**478**	**655**	**662**	**579**	**505**	**663**	6573	
Total percentage				**11.47**	**12.7**	**7.98**	**10.62**	**6.75**	**9.25**	**9.35**	**8.18**	**7.13**	**9.36**		**100**
OTUs per condition and sampling point				50	29	22	36	14	33	17	27	26	19		
Total OTUs				117		97		78		81		83			

3 CAPÍTULO II

3.1 Prueba *in vitro* para identificar potenciales antagonistas

3.1.1 INTRODUCCIÓN

En la agricultura moderna, se ha eludido la sostenibilidad de la productividad agrícola. El uso de agroquímicos ha permitido obtener incrementos substanciales en la producción; no obstante, sus efectos están impactando negativamente la sostenibilidad de la agricultura. La práctica del monocultivo y la contaminación por el uso indiscriminado de agroquímicos han reducido la biodiversidad de los agroecosistemas, causando la inestabilidad de los mismos, la cual se manifiesta, entre otros efectos nocivos, en una mayor incidencia de plagas y enfermedades en los cultivos (Zavaleta-Mejia, 1999).

Sin embargo, un manejo ambientalmente sano y racional de las enfermedades de nuestros cultivos se podrá lograr, primero, aceptando que nuestro objetivo principal no debe de ser el de eliminar al patógeno responsable de la enfermedad sino más bien que a pesar de su presencia logremos obtener rendimientos económicamente redituables para el agricultor (Zavaleta-Mejia, 1999). Estas ideas han conducido a la búsqueda y establecimiento de nuevas estrategias que permitan el manejo de plagas y enfermedades. Una de estas estrategias es el uso de microorganismos nativos (procariotas) para el biocontrol de enfermedades tales como la causada por *Fusarium verticillioides*.

El desarrollo de técnicas adecuadas para evaluar posibles agentes de biocontrol es un paso crítico en el desarrollo de productos agrobiológicos, pues el éxito de todas las etapas posteriores, dependerá de la elección de una apropiada metodología para la selección de un candidato apropiado (Cavaglieri *et al.*, 2004).

El poseer una colección de microorganismos identificados molecularmente y una metodología automatizada que nos permita su rápida manipulación y escrutinio, brinda la posibilidad de ofrecer una capacidad de respuesta más rápida y eficiente, para desarrollar productos agrobiológicos para el control de *Fusarium* y de otros fitopatógenos en la región, que ataquen a maíz u otros cultivos comerciales.

Por lo anterior, en el presente trabajo, se desarrolló una técnica que nos permite cuantificar la disminución de la masa fúngica en presencia de potenciales agentes de biocontrol, con capacidad de probar un gran número de muestras de una manera rápida, en poco espacio y de manera automatizada. El siguiente anexo responde al segundo objetivo planteado en el trabajo de tesis: estandarización de la metodología de monitoreo masivo *in vitro* de la colección de microorganismos para seleccionar antagonistas potenciales contra *Fusarium verticillioides*.

3.1.2 ANTECEDENTES

La pared celular de los hongos es una estructura con gran plasticidad, que da la forma a la célula, controla la permeabilidad celular y protege a la célula de los cambios osmóticos. Además de estas importantes funciones, constituye el lugar de interacción con el medio externo, localizándose en ella las adhesinas y un gran número de receptores que, tras su activación, desencadenarán una compleja cascada de señales en el interior de la célula (Pontón, 2008). La pared es el primer lugar de interacción entre el patógeno y su hospedador y juega un papel muy importante en la patogénesis fúngica (Chaffin *et al.*, 1998; Nimrichter *et al.*, 2005; Pontón, 2008), por lo que su eliminación o los defectos en su formación tienen efectos profundos en el crecimiento y la morfología de la célula fúngica, pudiendo causar la muerte celular por lisis (Heitman, 2005). La pared fúngica está compuesta básicamente de polisacáridos y proteínas. Entre los polisacáridos destacan la quitina, el glucano y el manano ó el galactomanano. Por todo lo anterior, la pared celular es considerada un blanco muy importante para la acción de los fármacos antifúngicos (Heitman, 2005).

La aglutinina de germen de trigo (WGA, por sus siglas en inglés Wheat Germ Agglutinin) es un miembro bien caracterizado de la clase aglutinina vinculante (lectinas) a quitina de la familia Poaceae (Goldstein y Hayes, 1978). Cuando el WGA es conjugado con moléculas fluorescentes (como el Alexa-Fluor), puede facilitar la localización de tejido fúngico en muestras complejas, como lo son las micorrizas arbusculares (Javot *et al.*, 2007), y hongos patógenos de plantas infectando el tejido vegetal (Galindo-Flores *et al.*, 2005).

En el siguiente anexo se describe la validación del empleo de WGA acoplada a un fluoróforo para cuantificar biomasa fúngica en un bioensayo que puede ser automatizable y que permite un rápido escrutinio en un ensayo en líquido retando aislados bacterianos contra el hongo *Fv* para ubicar potenciales bacterias antagonistas a este fitopatógeno.

3.1.3 ANEXO 2 (Artículo en prensa en la revista Journal of Basic Microbiology. DOI 10.1002/jobm.201200594)

Method Paper

A high-throughput screening assay to identify bacterial antagonists against *Fusarium verticillioides*

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KEYWORDS: WGA, Alexa Fluor® 488 conjugate / High-throughput screen / Fluorescence bioassay / Bacterial antagonism / Confocal Laser Scanning Microscopy.

ABSTRACT

A high-throughput antagonistic assay was developed to screen for bacterial isolates capable of controlling the maize fungal phytopathogen *Fusarium verticillioides*. This assay combines a straightforward methodology, in which the fungus is challenged with bacterial isolates in liquid medium, with a novel approach that uses the plant lectin wheat germ agglutinin (WGA) coupled to a fluorophore (Alexa-Fluor® 488) under the commercial name of WGA, Alexa Fluor® 488 conjugate. The assay is performed in a 96-well plate format, which reduces the required laboratory space and streamlines quantitation and automation of the process, making it fast and accurate. The basis of our assay is that fungal biomass can be assessed by WGA, Alexa Fluor® 488 conjugate staining, which recognizes the chitin in the fungal cell wall and thus permits the identification of potential antagonistic bacteria that inhibit fungal growth. This principle was validated by chitin-competition binding assays against WGA, Alexa Fluor® 488 conjugate; confocal laser microscopy confirmed that the fluorescent WGA, Alexa Fluor® 488 conjugate binds to the chitin of the fungal cell wall. The majority of bacterial isolates did not bind to the WGA, Alexa Fluor® 488 conjugate. Furthermore, including washing steps significantly reduced any bacterial staining to background levels, even in the rare cases where bacterial isolates were capable of binding to WGA. Confirmatory conventional agar plate antagonistic assays were also conducted to validate our technique. We are now successfully employing this large-scale antagonistic assay as a pre-screening step for potential fungal antagonists in extensive bacteria collections (on the order of thousands of isolates).

Maize (*Zea mays* L.) is one of the four basic food staples that support the world's population [1]. Although developed countries mainly produce maize for animal feed and industrial uses [2], maize represents a major food source in Mexico [3]. The majority of the reported maize diseases which affect roots, stalks, ears, and kernels are caused by fungi [4]; this includes 'stalk, root and ear rot', one of the most important diseases in countries which grow maize. In Mexico, the species that have been found to cause this disease are *Fusarium verticillioides* [5] and *F. subglutinans* [6, 7]. Stalk, root and ear rot is the most severe agricultural disease in Mexico's central Highland Valley area, as it lowers both yield and grain quality [8].

Our approach to this problem is to find novel antagonistic bacteria against fungal phytopathogens. However, screening for individual isolates from large collections (containing hundreds or even thousands of isolates) can be challenging, as this generally involves time-consuming microbiological techniques and necessitates dedicated laboratory space. Another constraint is that antagonism assays traditionally require solid medium plates to grow both the bacterial antagonists and pathogens, for dual culture assays [9, 10, 11]. The objective of this study was to develop a high-throughput methodology to rapidly screen large numbers of bacterial isolates in the search for antagonists against *Fusarium verticillioides* (*Fv*), the causative agent of the stalk, ear and root rot of corn [12, 13], which is a serious disease afflicting Mexico's maize production [14]. High-throughput screening using bioluminescent/fluorescent assays has mainly been developed and utilized for finding pharmacological targets for drug discovery [15, 16, 17, 18]. To the best of our knowledge, our study is the first of its kind to apply high-throughput fluorescence assays in order to discover novel bacterial antagonists against fungal phytopathogens, although several reports have developed high-throughput screening using different approaches, for biocontrol purposes [19, 20].

Wheat germ agglutinin (WGA) is a well-characterized member of the chitin-binding class of lectins from the Poaceae family [21], which specifically binds to N-acetyl-D-glucosamine and N-acetyl-D-neuraminic (sialic) acid residues [22, 23, 24]. When conjugated to fluorescent molecules (such as Alexa-Fluor, tetramethylrhodamine or fluorescein conjugates, *etc.*), WGA can facilitate the localization of fungal tissue in complex samples, such as the arbuscular mycorrhizal fungi that colonize roots [25], and plant pathogenic fungi that infect plant tissues [26]. In our novel antagonism assay this fluorescent WGA, Alexa Fluor® 488 conjugate (Cat. No. W11261, Life Technologies, Eugene, OR, USA) eliminates any subjective measurements of fungal growth inhibition.

In our screening assay, fungal biomass is quantified as relative fluorescence units due to the specific binding of WGA, Alexa Fluor® 488 conjugate to different chitin residues of the fungal cell wall. To evaluate WGA, Alexa Fluor® 488 conjugate efficiency of binding to purified chitin, we prepared colloidal chitin (Cat C-7170, St. Louis, MO, USA) according to Shanmugaiah [27]. Adding increasing amounts of chitin to 0.5 mL of phosphate buffer saline (1X PBS)

containing 0.5 µg of WGA, Alexa Fluor® 488 conjugate induced the precipitation of a chitin-WGA complex after overnight incubation at 4°C. This complex was removed by centrifugation at 17,900 x g. Subsequently, fluorescence of the supernatant was evaluated with a DTX880 multimodal detector (Beckman Coulter, Brea, CA, USA) using the EX1 filter slide for excitation (485-535 nm wavelength) and the EMP1 filter slide for emission (465-625 nm wavelength). A blank tube containing 1X PBS and Alexa Fluor® 488 conjugate was used to set up a background control. This tube was also incubated overnight at 4°C and washed three times with 1X PBS by centrifugation and then used for fluorescence counting. The remaining fluorescence from the supernatant corresponds to the amount of unbound WGA, Alexa Fluor® 488 conjugate (as compared to the original fluorescence level). Ten µg of colloidal chitin reduced the fluorescence of the supernatant by 80% (*i.e.* about 80% of the fluorescent WGA, Alexa Fluor® 488 conjugate was bound); amounts from 30 to 50 µg reduced it by more than 90% (Supplementary material Figure S1). This result is consistent with the WGA, Alexa Fluor® 488 conjugate binding to chitin residues of the fungal cell wall.

For the *Fv*/bacteria antagonistic assays, we used a cryopreserved monoconidial *Fv* culture whose symptomatology, pathogenicity and molecular identity were previously characterized by our group. The frozen stock (-70 °C) was used as a starter culture, and *Fv* was grown on potato dextrose agar (PDA; Cat. No. 213400, BD Difco, Franklin Lakes, NJ, USA) plates for seven to ten days at 25 ºC. Subsequently, a mycelium plug (0.5 cm in both diameter and height) was placed in a new PDA plate for seven days. *Fv* mycelia-containing conidia were resuspended in sterile distilled water and counted (Neubauer chamber) to adjust the conidia concentration.

A dose-response experiment was conducted to determine the amount of WGA, Alexa Fluor® 488 conjugate necessary to measure fungal biomass in 96-well plates, in which each well contained 0.5 mL of potato dextrose (PD) broth (Cat. No. 254920 BD Difco, Franklin Lakes, NJ, USA) (Supplementary material Figure S2). *Fv* inoculum (2 x 10^5 conidia mL^{-1}) was grown at 25°C for 36 h, and the fungal biomass collected by centrifugation at 17,900 x g. Fluorescence associated to fungal biomass was quantitated after staining by overnight incubation at 4°C with 1X PBS added with WGA, Alexa Fluor® 488 conjugate ranging from 0.2 to 2.5 µg mL^{-1}, and followed by washing three times with 1X PBS and centrifugation. A blank tube containing 1X

PBS and Alexa Fluor® 488 conjugate was used to set up a background control. It was incubated overnight at 4°C and also washed three times with 1X PBS by centrifugation and then used for fluorescence measurements. Five replicates were quantified per each dosage amount used. The fluorescence values for WGA, Alexa Fluor® 488 conjugate were similar when either a 2.5 or a 1 or 2 µg mL^{-1} doses were used. We selected 1 µg mL^{-1} of WGA, Alexa Fluor® 488 conjugate for further experiments, which is in accordance with the manufacturer's recommendations.

We next examined the influence of initial inoculum size on fungal biomass. Growth kinetics showed that the different conidia concentrations followed a similar trend (Figure 1A). At 36 h of growth, the 2 x 10^5 and 2 x 10^6 conidia mL^{-1} concentrations showed similar growth patterns; by contrast, at 2 x 10^4 conidia mL^{-1}, *Fv* growth was significantly reduced as compared to the 2 x 10^5 conidia mL^{-1} condition. No significant differences were observed between different inoculum concentrations after 48 and 72 h of growth, and by 72 h a decrease in fluorescence was detected for both the 2 x 10^4 and 2 x 10^6 conidia mL^{-1} concentrations. This decrease may be due to excessive fungal growth that was not properly centrifuged, as well as manipulation of the fungal tissue. It is also possible that fungal material could change its cell wall structure during different growth stages, which could prevent WGA binding. Based on the obtained results, and to minimize changes to fungal cell wall structure that could affect fluorescence measurements (due to fungal tissue aging), we decided for the screening assay protocol to fix fungal growth at time 36 h and to add 2 x 10^5 mL^{-1} conidia as the initial inoculum.

Fungal growth kinetics are difficult to follow in a 96-well plate format, given the very small amount of fungal dry biomass that can be recovered from 0.5 mL of PD broth. For this reason, we only weighed fungal biomass at a late time point (72 h of growth), and could determine the maximal range of biomass growth (measured as dry biomass). Values for dry fungal tissues from different experiments ranged from 0.8 to 1.2 mg. To verify that fungal biomass can be accurately measured (at least up to 1.2 mg) using the WGA, Alexa Fluor® 488 conjugate, we grew the fungus for 72 h in 100 mL of PD broth, after which fungal tissue was collected and dried at 100°C until constant weight was obtained (approximately 12 to 16 h). Dry fungal tissue was ground to a fine powder using a porcelain mortar and pestle, and was similarly desiccated to a constant dry weight. A suspension of dry fungal biomass was prepared,

ranging from 0.2 to 4 mg in 0.5 mL of PBS buffer. The fungal tissue was stained with WGA, Alexa Fluor® 488 conjugate (1 µg mL^{-1}) and quantitated as described above. A correlation between fungal biomass and fluorescence count was observed from 0.2 to 1.2 mg fungal dry weight, confirming that fluorescence count accurately reflects fungal biomass in this assay, when the selected 1 µg mL^{-1} concentration of WGA, Alexa Fluor® 488 conjugate is used (Figure 1B). Fungal biomass higher than 1.2 mg could not be accurately measured, as saturation of the WGA, Alexa Fluor® 488 conjugate (reaching a plateau) in fluorescence counts was observed when we used up to 4 mg of fungal tissue (inset Figure 1B). By performing the antagonistic *Fv*/bacteria assays at 36 h we assure that fluorescence will be accurately measured, and that fungal biomasses higher than 1.2 mg will not be reached in our assays, even in the remote case that some bacteria will cause growth promotion of the fungus.

The bacterial isolates in this liquid antagonistic assay belong to a cryopreserved (-70°C) collection of 11,520 bacteria isolates from the maize rhizosphere (Scientific Collection CIIDIR-003), arranged in a 96-well plate format. The plate containing the stock isolates was handled in a laminar hood. Sterile 200 µl tips were carefully placed inside each well of the plate, and the tips were scraped against the frozen glycerol stocks to load inside a small amount of melted bacterial suspension. Subsequently, 500 µl of PD broth were aliquoted to each well of a 2 ml 96-well plate (Cat. No. 140504 Beckman Coulter, Brea, CA, USA) and inoculated with the tips containing the bacterial isolates. Finally, each well received 2 x 10^5 conidia mL^{-1}. The plates were covered with a sterile aluminum seal and incubated at 25 ºC and 250 rpm for 36 h in an orbital shaker. After incubation, the 96-well plates were centrifuged at 6,570 x g for 10 min in a Beckman J-30I centrifuge using a swinging-bucket rotor (JS 5.9, Beckman Coulter) and the supernatant was discarded. The mycelial pellet was resuspended and washed four times by centrifugation with 0.5 ml of 1X PBS solution (with a final concentration of 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, and pH 7.4). The pellet was then resuspended in 0.5 ml of 1X PBS, and 0.5 µg of WGA, Alexa Fluor® 488 conjugate was added to obtain a final concentration of 1 µg mL^{-1}. Following overnight incubation at 4ºC, the plate was centrifuged to remove the supernatant and the pellet was washed four times (in 1 ml 1X PBS) by centrifugation to eliminate residual WGA, Alexa Fluor® 488 conjugate and bacteria. After the final wash, the pellet was resuspended in 0.2 ml of 1X PBS and placed in an optical 96-well plate (Cat. No.

609844, Beckman Coulter, Brea, California, USA). Subsequently, fluorescence of the fungal pellet was counted as described before. This protocol facilitates handling sets of two or four 96-well plates, depending on the type of centrifuge rotor available, to the point where four to eight 96-well plates can easily be processed in two eight-hour work days by a single person. This workload can be improved when using robotic liquid-handling platforms under sterile conditions.

To verify whether the presence of bacteria could influence the WGA-based fungal staining assay, we first checked whether the WGA, Alexa Fluor® 488 conjugate could bind to the bacteria. In bacteria cultivated alone, we determined by confocal laser scanning using a white laser in a TCS SP5X microscope (Leica) that WGA, Alexa Fluor® 488 conjugate binds to some isolates either at the extracellular matrix surrounding the cell wall (Supplemental Material Figure S3) or to their cell walls (Figure 2A). It is possible that isolates showing fluorescence are gram-positive, since their bacterial cell wall has N-acetylglucosamine residues and they are recognized by WGA, Alexa Fluor® 488 conjugate [28]. In the case of gram-negative bacteria, WGA, Alexa Fluor® 488 conjugate should not be able to penetrate the outer membrane and thus could not attach to the peptidoglycan. Nevertheless, we verified that the final bacterial load is greatly reduced in our assay, thanks to the many washes included in the protocol. WGA, Alexa Fluor® 488 conjugate staining and washing of 96-well plates that contain the bacteria-only control revealed no significant fluorescence (Figures 2E and 2I, see "bact" bar). These results suggest that bacteria (regardless of their ability to bind WGA) are effectively washed during the assay. Any bacteria remaining after washing will thus have a negligible effect on fluorescence counts, and should therefore not be considered in analyzing the results.

We also confirmed visually, by scanning confocal laser microscopy, that the fungal cell wall is stained with WGA, Alexa Fluor® 488 conjugate when the fungus is grown alone (Figure 2B and F), and when it is grown in the presence of antagonistic bacteria (Figure 2C and G). By adding excess chitin to *Fv* grown alone and allowing it to compete for the chitin residues of the fungal cell wall, we demonstrated that chitin will bind to the WGA, Alexa Fluor® 488 conjugate; this prevents the lectin moiety from binding to the fungal cell wall, and it is subsequently removed by washing (Figure 2D and H). Excess chitin forms small precipitates that are fluorescent green and that are not easily removed by washing (see inset close-up of Figure 2D), although no staining of the fungal hyphae was observed. This demonstrates that WGA, Alexa

Fluor® 488 conjugate indeed acted directly in the assay by binding to the fungal cell wall. Visual observations were corroborated by fluorescence measurements, which indicated that the fungus was solely responsible for fluorescence counts in this procedure, and not the bacteria (Figure 2I).

The decrease in fungal biomass caused by the antagonistic bacterial isolate was measured as a decrease in fluorescence as compared to the 'fungus only' control. To evaluate this change, we calculated the percentage of fungal growth as follows: Fwell = Ff + Fb + Fblank where Fwell equals to the fluorescence of the well which is the sum of the fluorescence of the fungus (Ff) plus the fluorescence of the remaining bacteria after washing (Fb) and the fluorescence of the blank (Fblank). Since Fb is negligible (Fig 2), you can ignore it, and Fwell = Ff + Fblank. Then, we get: Ff = Fwell – Fblank. This calculation is true for all the wells (with or without bacteria), and the same Fblank value was substracted to all values. Please note that it should also be removed from the Control well (fungus only). To calculate the decrease in fungal biomass caused by the antagonistic bacterial isolate, the Ff value of the sample (Ff_sample) is compared to the Ff value of the control (Ff_control), and is expressed as a percentage of Ff_control. This gives: Fungal growth (% of control) (%FG) = Ff_sample x 100 / Ff_control.

The percentage of fungal growth inhibition (%FGI) was calculated by subtracting the percentage of fungal growth (%FG) from 100 (*i.e.* %FGI = 100 - %FG). The criterion used to select a good antagonist was arbitrarily set to >60% growth inhibition. We did not perform growth kinetics for each isolate, as this is typically an unmanageable task in such a massive screen. In the present work, we did perform bacterial growth kinetics for several isolates (data not shown) and observed that they reached the stationary phase by 36 h. The conditions used in this assay, such as PD broth selection, are biased to favor fungal growth. Based on the assumption that this would enable finding bacterial isolates that are antagonists against *Fv*, we performed a test with 96 isolates (Figure 3A). One plate was randomly selected from the Scientific Collection CIIDIR-003 and tested as previously described for the liquid antagonism assay. Thirty-six isolates exhibiting >60% *Fv* growth inhibition were identified (Figure 3A), and subsequently their potential antagonistic activity was tested in a 96-well solid medium antagonistic assay. For this assay, isolates were defrosted and grown in 2 mL deep-well 96-well plates in 0.5 mL of LB broth (Cat. L3022 Sigma, St Louis, MO, USA) overnight at 25 °C and 200

rpm. Bacterial pellets were then picked using a multichannel micropipette (8 channels). In order to inoculate the bacteria on 96-well plates filled with 200 µl of PDA solid medium, the pellet was touched with a 10 µL tip to deposit the bacteria on the right side of each well. After bacterial inoculation of each well of the plate, 2 µL of water containing 1 x 10^4 *Fv* conidia were deposited in the left side of the well. In this assay, *Fv* was challenged with the bacterial isolates for 2 days in PDA medium cultured at 25 °C in 96-well agar plates (Figure 3B), performed in triplicate. All thirty-six isolates exhibiting >60% *Fv* growth inhibition were subsequently tested in this assay, as well as a confirmatory assay using a conventional agar plate antagonist assay (data not shown). Three out of the thirty-six isolates were confirmed as growth inhibitors of *Fv* (Figure 3B). This diminished number of isolates is consistent with the view that the liquid medium assay is a preliminary screen, and follow up confirmation with other assays such as dual culture analysis is just one step in the selection procedure for antagonists of a fungal pathogen. In our experience with extensive collections of rhizospheric organisms, *in vitro* plate screening for antagonists can diminish the number of potential antagonists to less than 10% of the original isolate number [29]. Since dual culture analysis is normally performed in 50 – 100 mm diameter Petri plates, we conducted confirmatory experiments that gave similar results for fungal growth inhibition (see supplementary material Figure S4). The isolate in Figure S4B corresponds to isolate F8 from Figure 3A exhibiting 69% growth inhibition in the 96-well plate assay. In contrast to the 96-well plate system, using a 50 mm diameter Petri plate resulted in a lower percentage of *Fv* growth inhibition (39%). In general, this was observed with all other isolates tested by the conventional Petri plate assay. This trend can be explained by considering that this type of dual culture assay allows screening for fungal growth inhibition mechanisms where diffusion of secreted inhibitory compounds is involved. It is possible that the closer proximity between the two organisms in the 96-well plates (only 8 mm in diameter) allows for a more rapid diffusion of these substances and for faster growth inhibition responses than in the larger volume Petri plates.

Antagonistic bacteria secrete diverse compounds that can act upon fungal pathogens, and as such, various antagonistic mechanisms can be detected in the liquid assay. These compounds can affect either spore germination [30] or elongation and growth of hyphae; in *Bacillus* spp. these effects are due to the action of enzymes such as chitinases [31, 32], or

antibiotics such as kanosamine and zwittermicin A [33, 34]. In the case of this particular assay where the fungus and bacteria are in direct contact, another plausible antagonistic mechanism could involve mycoparasitism [35]. *In vitro* screening only allows selection which must be confirmed *in planta*, where the tripartite interaction occurs between the antagonistic bacteria, the fungus and the plant root [36].

There has been a great focus on developing techniques to identify antagonists that inhibit plant pathogens, in an effort to control disease in crops of interest such as corn [32, 37, 38]. The present methodology was designed to explore the antagonistic ability of a collection of 11,520 native bacterial isolates from the rhizosphere of cultivated maize in Sinaloa, Mexico (unpublished work). Our protocol is ideal for performing an antagonism bioassay with large numbers of specimens, and its advantages include less required laboratory/incubator space coupled with a shorter assay period, as well as the possibility of automation using liquid handling robotic platforms. We have demonstrated that this assay is suitable for screening large collections of bacteria in search of potential antagonists against fungal plant pathogens, as well as other types of fungal pathogens. It is always advisable to perform a small test of the liquid *Fv*/bacteria antagonistic assay in duplicates or triplicates to corroborate repetitiveness of the assay before starting a massive screening procedure. Nevertheless, performing this assay in duplicates or triplicates has to be evaluated by the researchers considering the size of the screening, time and/or budget constraints. In our experience, a combination of the massive liquid assay with confirmatory tests would work the best. Our high-throughput screening assay, in combination with dual culture analysis and other screening procedures (including *in planta* confirmatory bioassays), has allowed our group to select potential bacterial antagonists against the stalk, root and ear rot disease in maize. Preliminary results for these antagonists in field trials show promising results for the biological control of *F. verticillioides* in maize (manuscript in preparation).

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Figure legends

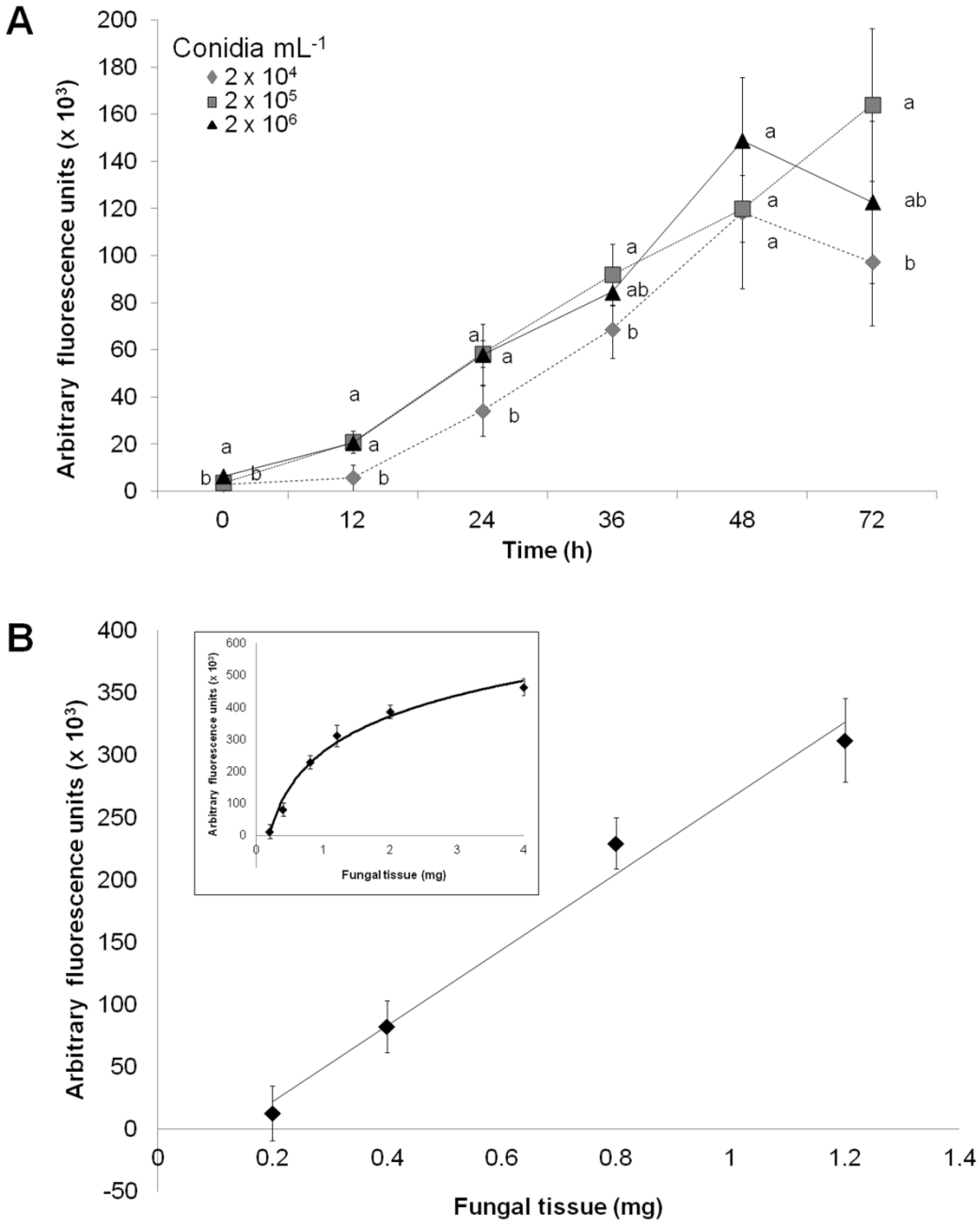
Figure 1. A) Growth kinetics of *Fv* in PD broth using different conidia concentrations. For detection of fungal growth as relative fluorescence units, five replicates were used per point. Tissue was collected at different time points by centrifugation and transferred to a 96-well optical plate to be stained with 1 µg mL^{-1} of WGA, Alexa Fluor® 488 conjugate by overnight incubation at 4°C. Fluorescence was counted after washing three times by centrifugation. Initial conidia concentrations are indicated by diamonds (2 x 10^4 conidia mL^{-1}), squares (2 x 10^5 conidia mL^{-1}) and triangles (2 x 10^6 conidia mL^{-1}). Different letters indicate significant differences (P = 0.05) between values measured at a given time point. B) WGA, Alexa Fluor® 488 conjugate fluorescence counts correlate with fungal biomass. Increasing amounts of fungal biomass (dry tissue) were stained using 1 µg mL^{-1} WGA, Alexa Fluor® 488 conjugate. The correlation coefficient (R) was 0.9839. The inset shows that at higher amounts of fungal biomass WGA, Alexa Fluor® 488 conjugate fluorescence counts reach saturation. Each point represents the average of five replicates (diamonds) and bars indicate standard deviation.

Figure 2. Dual WGA, Alexa Fluor® 488 conjugate and propidium iodide staining revealed that lectin binds to the cell walls of the fungus and bacteria. Merged images are shown in panels A – H. A Leica TCS SP5 X confocal laser scanning microscope was used to obtain the two merged acquisition channels using 497 and 489 nm excitation laser (white laser), and emission ranges of 502-548 nm and 598-706 nm for WGA, Alexa Fluor® 488 conjugate (green fluorescence) and for propidium iodide (red fluorescence), respectively. A) Close-up of bacterial cells showing WGA binding to the cell walls. E) Last wash following WGA, Alexa-Fluor® 488 conjugate staining from a well of bacteria-only control, illustrating that the remaining bacterial cells are greatly reduced in number. B and F) *Fv* fungal mycelium grown alone and stained with WGA, Alexa Fluor® 488 conjugate showing green fluorescence in their cell walls. C and G) *Fv* fungal mycelium grown with bacteria in an antagonistic assay and stained with WGA, Alexa Fluor® 488 conjugate showing green fluorescence in their cell walls. D and H) *Fv* fungal mycelium stained with WGA, Alexa Fluor® 488 conjugate in the presence of excess colloidal chitin (50 µg) reveals that competition with chitin prevents lectin from binding to the fungal cell wall, as indicated by the absence of green fluorescence. The inset in D indicates that the green spots observed in

this image are possibly chitin precipitates bound to WGA, Alexa Fluor® 488 conjugate. I) Quantitation of fluorescence after the series of washes demonstrates that any remaining bacteria are not responsible for fluorescence in the antagonistic assay, and that the green fluorescent stain is due to the binding of WGA, Alexa Fluor® 488 conjugate to N-acetyl glucosamine residues. Bact stands for bacteria; *Fv* indicates *F. verticillioides* grown alone. *Fv* + Bact is the antagonistic assay in which *Fv* and bacteria are incubated together. Under this condition, the bacteria have an antifungal activity; similar results are obtained with bacteria that do not inhibit fungal growth only that fluorescence counts will change. *Fv* + Chitin refers to a binding assay between chitin and WGA, followed by the staining of the fungus grown alone (set up as a lectin-chitin binding control). The same antagonistic bacterium was used for panels A, C, E, G and I.

Figure 3. Bacterial antagonism assay against *Fv* in 96-well plates. A) Percentage of growth inhibition caused by 96 bacterial isolates against *Fv* in PD broth (liquid antagonism assay). Negative values denote promotion instead of inhibition of fungal growth by those specific bacterial isolates. The gray boxes indicate isolates showing higher than 60% fungal growth inhibition that were selected for further confirmation as potential antagonists, while white boxes indicate ≤60% growth inhibition. B) 96-well plate antagonism assays against *Fv* in PDA solid medium for three selected isolates. Isolates were chosen on the basis of their fungal growth inhibition in liquid medium assays (indicated as a percentage to the right of their respective photographs), and correspond to A2, F4 and F8 from panel A. Photographs corresponding to only one well from different 96-well plates are displayed, with results shown in triplicate. *Fv* conidia were placed on the left side of each well and the bacterial isolates were placed on the right side of each well, except for the fungus-only control (CTL). Plates were evaluated after 48 h of incubation at 25 °C, at which point the fungal hyphae reached the right edge of the CTL well.

Figure 1


A
Conidia mL-1
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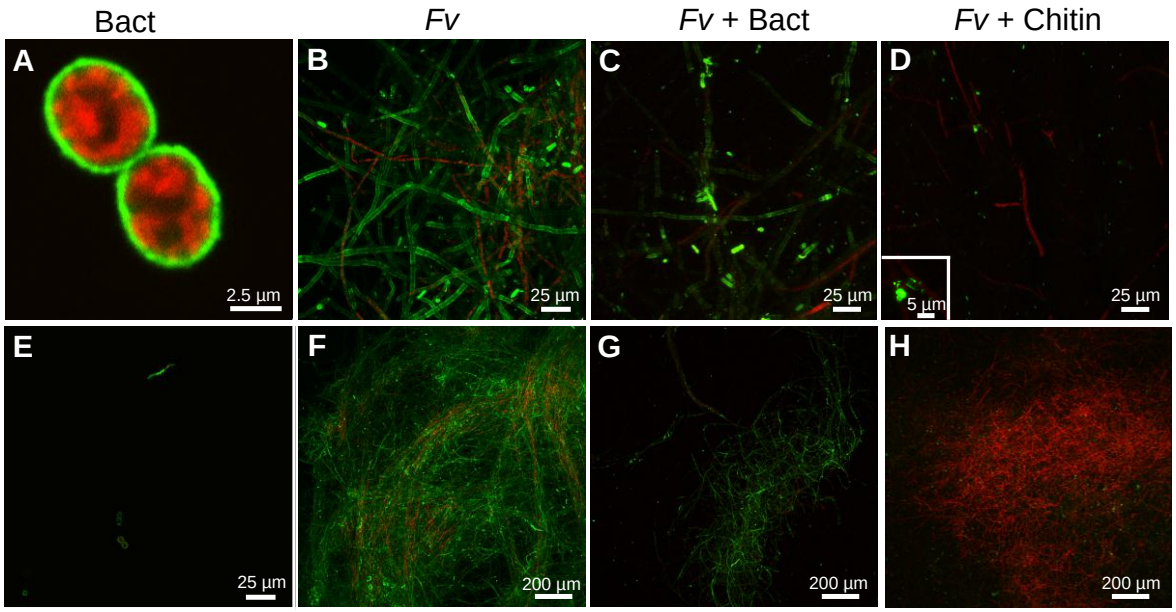
2 x 10⁵
2 x 10⁶
Arbitrary fluorescence units (x 10³)
Time (h)
0
12
24
36
48
72
B
Arbitrary fluorescence units (x 10³)
Fungal tissue (mg)

Figure 2


Bact
Fv
Fv + Bact
Fv + Chitin
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200 µm
200 µm

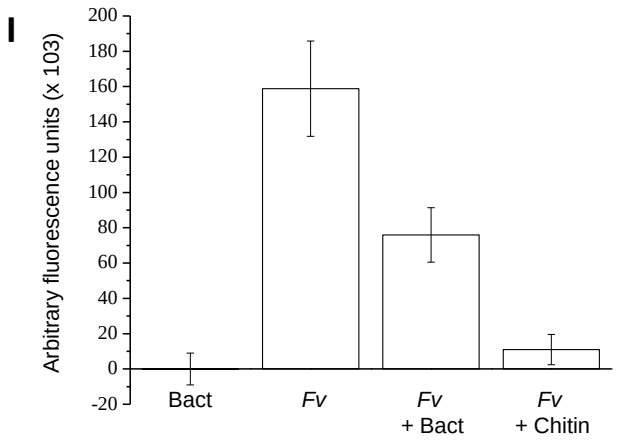

I
Arbitrary fluorescence units (x 103)
200
180
160
140
120
100
80
60
40
20
0
-20
Bact
Fv
Fv + Bact
Fv + Chitin

Figure 3

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	74	77	70	65	32	80	17	85	24	19	78	71
B	62	84	79	13	73	63	82	12	54	3	57	21
C	72	63	26	-19	88	51	78	62	11	31	-34	50
D	87	23	-13	84	81	44	21	31	27	16	82	23
E	51	24	-20	-23	55	3	-5	7	25	68	-7	-28
F	52	42	77	90	12	37	17	69	63	56	-47	56
G	39	62	51	65	68	7	61	49	34	99	51	50
H	46	51	50	46	46	-1	32	-66	12	71	65	74

B

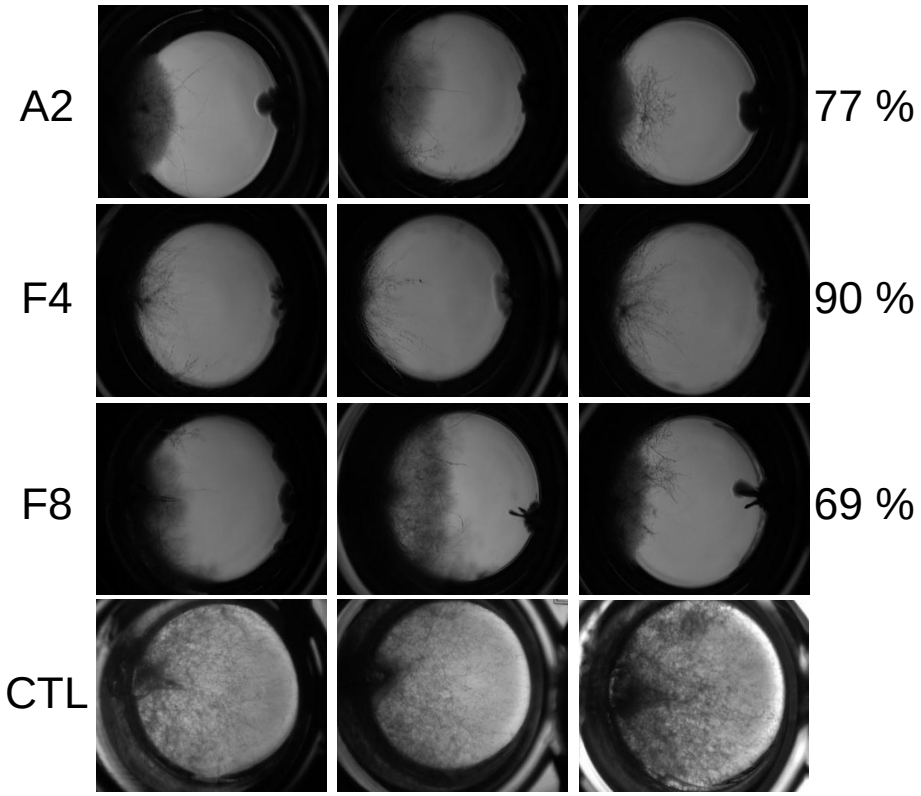


Figure legends for supplementary material

Figure S1. Chitin binding assay against WGA, Alexa Fluor® 488 conjugate. Different amounts of colloidal chitin were incubated with 1 µg mL-1 of WGA, Alexa Fluor® 488 conjugate, and were allowed to bind overnight at 4°C. Colloidal chitin bound to the WGA, Alexa Fluor® 488 conjugate was eliminated by centrifugation at 17,900 x g and the supernatant was used to quantitate fluorescence of free WGA, Alexa Fluor® 488 conjugate. Fluorescence units are expressed as a percentage relative to the WGA, Alexa Fluor® 488 conjugate solution without chitin.

Figure S2. Dose-response to establish the optimal amount of WGA, Alexa Fluor® 488 conjugate to use in the liquid antagonism bioassays. Different concentrations of WGA, Alexa Fluor® 488 conjugate were incubated overnight at 4°C with Fv fungal biomass, and the fluorescence of WGA, Alexa Fluor® 488 conjugate bound to the fungal cell wall was detected and expressed as relative fluorescence units. 2 x 105 conidia mL-1 were used as the initial inoculum and allowed to grow for 36 h at 25 °C before the fungal biomass was collected. Standard deviation bars are shown for each amount of WGA, Alexa Fluor® 488 conjugate used. Different letters indicate statistical differences between treatments (n=5) at a significance of P=0.05.

Figure S3. WGA, Alexa Fluor® 488 conjugate binds to bacterial cell walls. A bacterial isolate was grown for 36 hours in PD broth and harvested by centrifugation. The resulting pellets were stained either by resuspending them in PBS alone (A-C, without WGA) or PBS buffer containing the WGA, Alexa Fluor® 488 conjugate (D-F, with WGA). After overnight incubation at 4°C, bacteria were washed four times and the pellets were prepared for visualization by confocal laser scanning microscope (Leica TCS SP5 X) as described in Figure 2. For contrast, bacteria were counter-stained with the intracellular stain propidium iodide. A) Bacteria unexposed to WGA, Alexa Fluor® 488 conjugate do not exhibit any green fluorescence; B) Propidium iodide-stained bacteria show red fluorescence; C) Merged image of both channels; D) Bacteria stained with WGA, Alexa Fluor® 488 conjugate show green fluorescence located at the extracellular matrix/cell wall; E) Bacteria stained with WGA, Alexa Fluor® 488 conjugate and counter-stained

with propidium iodide show red fluorescence in the interior of the bacterial cells; F) Merged image of both beam lines in bacteria stained with WGA, Alexa Fluor® 488 conjugate and propidium iodide.

Figure S4. Bacteria selected as antagonistic against Fusarium verticillioides by high-throughput screening assays behave similarly in conventional dual culture assays. A) Control plate, where Fv is growing without any bacterial isolate. B) Positive control, in which bacterial isolate F8 (see Figure 1A and 1B) inhibits Fusarium growth. C) Negative control, in which bacterial isolate E6 (see Figure 1A) does not affect Fusarium growth. Arrow indicates the bacterial isolate position. Petri plates 50 mm in diameter were inoculated as described in the text for figure 3B for 96-well plate solid medium antagonistic assays and kept at 25°C for one week before photograph was taken.

Figure S1

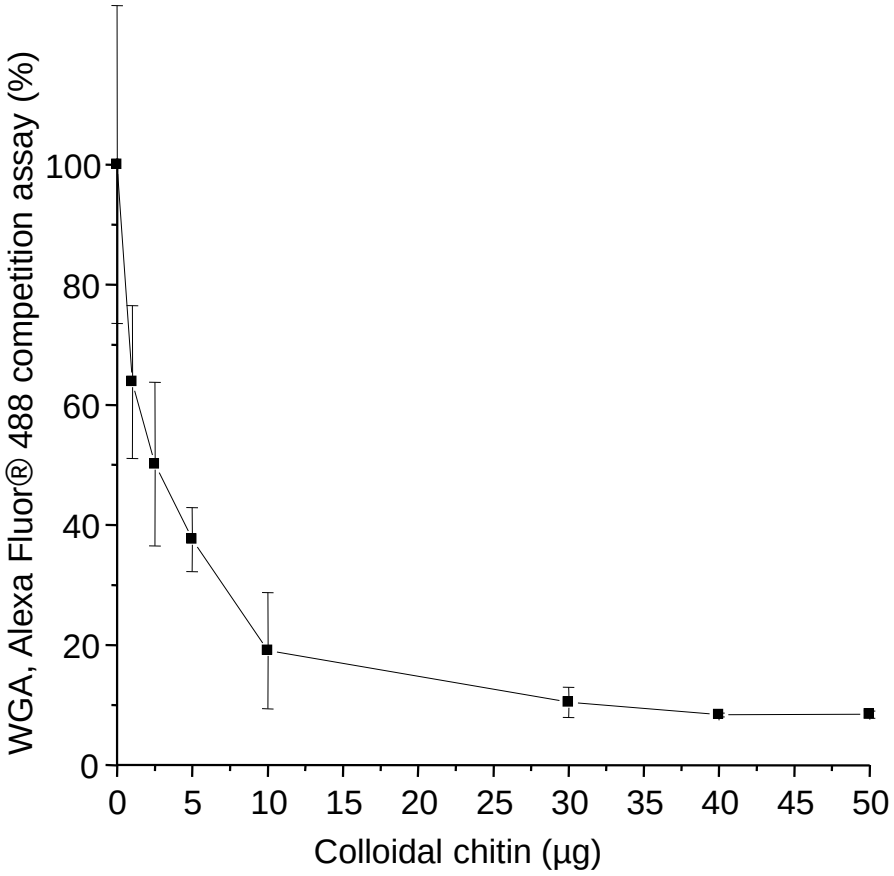

WGA, Alexa Fluor® 488 competition assay (%)
100
80
60
40
20
0
0
5
10
15
20
25
30
35
40
45
50
Colloidal chitin (µg)

Figure S2

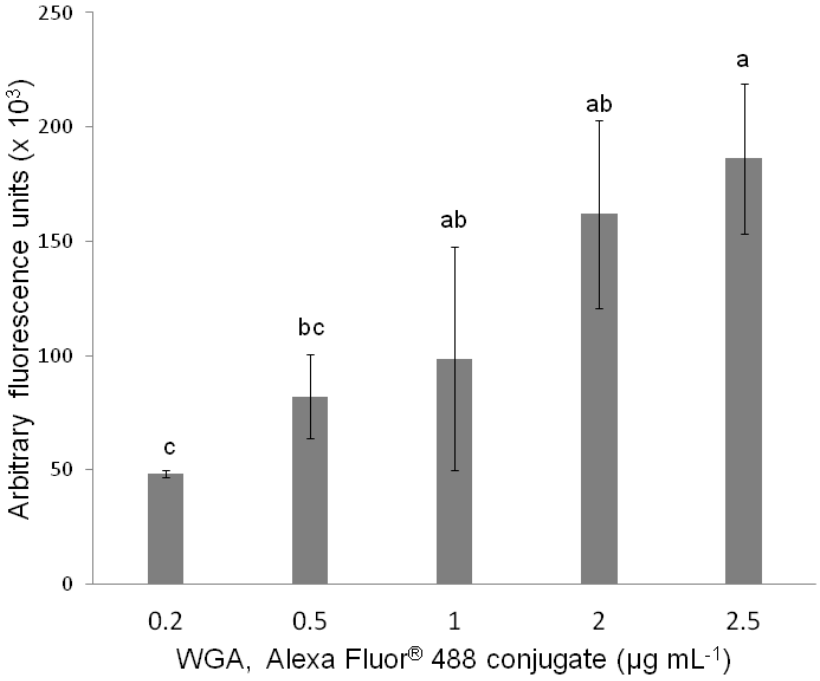

250
200
150
100
50
0
Arbitrary fluorescence units (X 10³)
c
bc
ab
ab
a
0.2
0.5
1
2
2.5
WGA, Alexa Fluor® 488 conjugate (µg mL⁻¹)

Figure S3

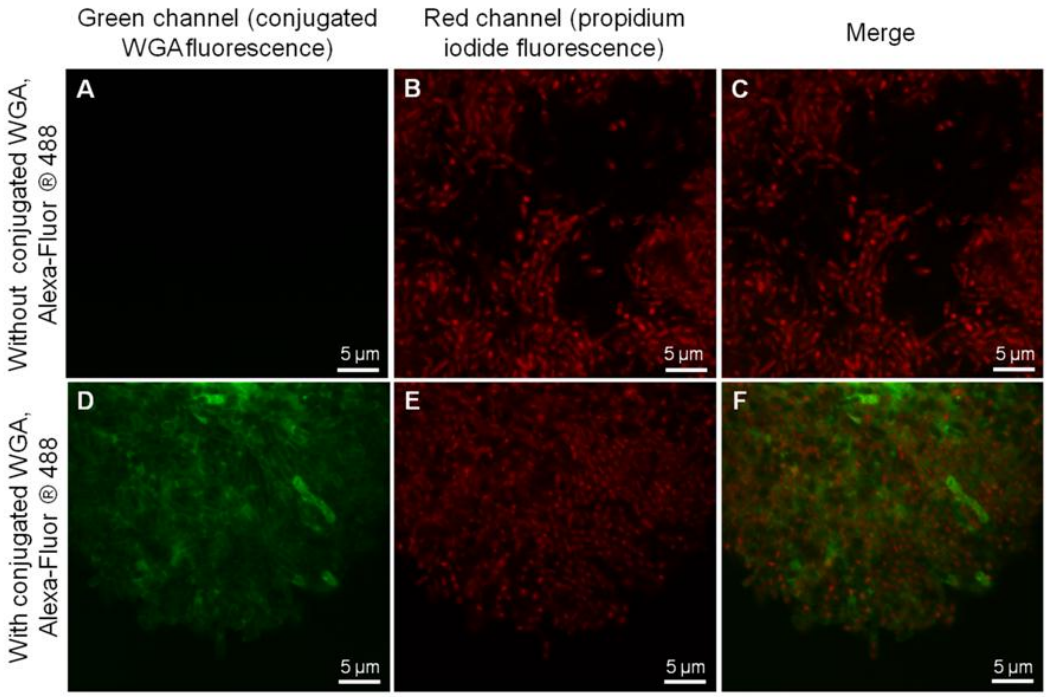
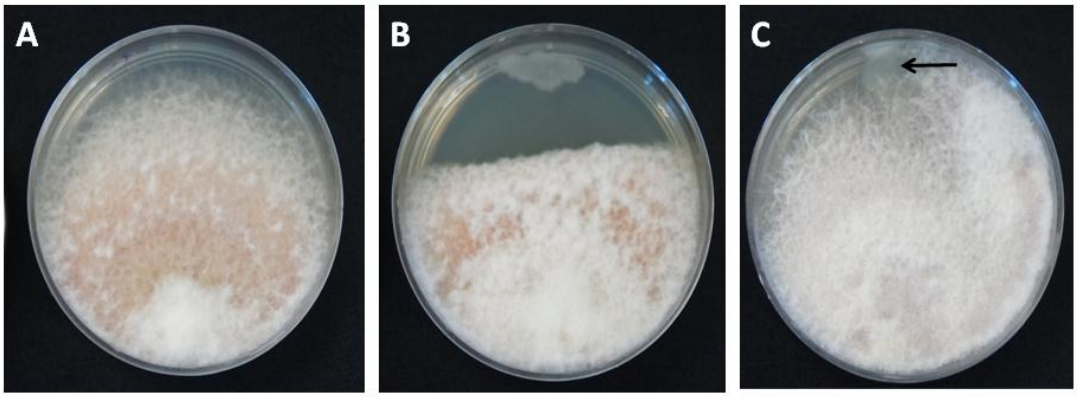

Green channel (conjugated WGA fluorescence)
Red channel (propidium iodide fluorescence)
Merge
Without conjugated WGA, Alexa-Fluor ® 488
With conjugated WGA, Alexa-Fluor ® 488
A
B
C
D
E
F
5 µm

Figure S4



4 CAPÍTULO III

4.1 Prueba *in planta* para identificar potenciales antagonistas de *Fusarium verticillioides.*

4.1.1 INTRODUCCIÓN

Entre los principales estados productores de maíz en México se encuentra Sinaloa, con una aportación del 18.41% del valor de producción total en el 2010. En ese período se sembraron 532, 791.14 Ha, de las cuales se cosecharon 525, 142.14 Ha con una producción de 5, 227, 872.02 Ton y un valor de $12, 086, 413, 890 de pesos (SIAP, 2010). En Sinaloa, el establecimiento del maíz como un monocultivo (Otoño-Invierno y Primavera-Verano), han propiciado la aparición y acentuación de enfermedades.

En el año 2006 se observó un aumento en la incidencia de pudrición del tallo y raíz en el Norte de Sinaloa y se especuló que la enfermedad se debía a *Fusarium*. La Junta Local de Sanidad Vegetal del Valle del Fuerte (JLSVVF) realizó un monitoreo en los municipios de Ahome y El Fuerte, durante 2006-2007. Se detectó a *Fusarium* en el 84% de las plantas de maíz inspeccionadas en el ciclo OI y en el 70% de las plantas inspeccionadas en el ciclo PV (Quintero-Benítez y Apodaca-Sánchez, 2008). En el ciclo PV 32% de plantas presentaron una alta severidad (superior a 40%), mientras que en OI fue de 13%. Las plantas afectadas mostraban marchitez y muerte del follaje, pudrición de la base del tallo, amarillamiento de las hojas inferiores, achaparramiento, proliferación de raíces aéreas, entre otros (Quintero-Benítez y Apodaca-Sánchez, 2008).

García-Pérez y Velarde (comunicación personal) analizaron varios lotes de maíz con síntomas de pudrición en plántulas y mazorcas de diferentes híbridos en la parte centro-sur del estado de Sinaloa, particularmente en el Valle de Culiacán. De dichos análisis, se obtuvieron 165 cepas monospóricas de *Fusarium*, a partir de tallos de plántulas, de las cuales, 140 se identificaron

molecularmente como *Fusarium oxysporum*. También se obtuvieron 92 cepas monospóricas a partir de mazorca, las cuales fueron identificadas como *Fusarium verticillioides*.

El control químico de éste patógeno se realiza a nivel de semilla antes de la siembra, sin embargo existen reportes de la inefectividad de los fungicidas empleados, así como incrementos significativos en las concentraciones de fumonisinas en plantas procedentes de semillas tratadas con agroquímicos así como en cultivos fúngicos adicionados con fungicidas (Pereira *et al.*, 2007b; Falcão *et al.*, 2010). Por ello, la tendencia actual es la búsqueda de agentes bioprotectores capaces de combatir las infecciones ocasionadas por *F. verticillioides* (Figueroa-López, 2011; Cordero-Ramírez *et al.*, 2012a; Leyva-Madrigal, 2013).

Por todo esto, se seleccionaron y evaluaron a los mejores aislados antagonistas de *F. verticillioides* resultantes de las pruebas *in vitro* en medio líquido y sólido, para evaluar la respuesta en planta de maíz a los potenciales antagonistas contra *F. verticillioides* con el fin de determinar cuáles pudieran ser los candidatos a probar en pruebas de invernadero y de campo.

4.1.2 ANTECEDENTES

El género *Fusarium* es uno de los patógenos más ubicuos, abundante e importantes de hongos microscópicos del suelo. El género contiene muchas especies de importancia para la salud ambiental, agrícola y humana, pero su relavancia biológica, se debe principalmente a la patogenicidad hacia una amplia gama de hospederos (Wakelin *et al.*, 2008).

Bacterias pertenecientes a los géneros *Pseudomonas, Bacillus, Burkholderia* y otros microorganismos del suelo han sido usados en experimentos en plantas con éxito para el biocontrol de *Fusarium,* tanto en la parte vegetativa como en el suelo (Kerry, 2000). Algunos biopesticidas comerciales incluyen bacterias que pertenecen a los géneros *Agrobacterium*, *Bacillus*, *Pseudomonas* y *Streptomyces*.

Aislados bacterianos obtenidos de rizósfera de maíz de los géneros *Burkholderia* y *Pseudomonas* fueron analizados por su capacidad de inhibir a *F. verticillioides*, en pruebas *in vitro* estos aislados inhiben el crecimiento del hongo en un rango del 38-68%, mientras que en pruebas *in vivo* se observó una inhibición del 66-88% (Hernández-Rodríguez *et al.*, 2008a). Un análisis metagenómico en remolacha azucarera (Mendes *et al.*, 2011), demostró que las poblaciones de bacterias pertenecientes a los phyla *Proteobacteria*, *Firmicutes* y *Actinobacteria* fueron los más dinámicos en relación a la supresividad del damping-off ocasionada por *Rhizoctonia solani*, y que miembros de la familia Pseudomonadaceae son los encargados de producir *in situ* antibióticos que controlan al fitopatógeno. En otros estudios, aislados de *Bacillus* obtenidos de rizósfera de plantas de maíz infectadas por *F. verticillioides* inhibieron 28-78%, y redujeron en 29-50% la producción de fumonisina B_1 (Cavaglieri *et al.*, 2005b)

En otros estudios, aislados de *Bacillus amyloliquefaciens* y *Microbacterium oleovorans* inhibieron el crecimiento de *F. verticillioides*, y disminuyeron la concentración de fumonisinas en granos de maíz (Pereira *et*

al., 2007b). De manera similar, cepas de *Azotobacter armeniacus* y *Arthrobacter globiformis* inhibieron 71-100% el crecimiento de *F. verticillioides in vitro* (Cavaglieri *et al.*, 2004).

Sin embargo, la selección de la herramienta adecuada para realizar el monitoreo de cepas nativas adaptadas localmente, es la clave para obtener un biocontrol adecuado en la mayoría de los casos (Cavaglieri *et al.*, 2004).

En el siguiente anexo se aborda el tercer objetivo del trabajo de tesis: la selección de los mejores antagonistas a *Fv* resultantes de las pruebas *in vitro* e *in planta*.

4.1.3 ANEXO III. Sometido a la revista Plant and Soil

Number of text pages: 34

Number of Tables: 2 tables and 1 supplementary table

Number of Figures: 4 figures

Short Running Title: Rhizospheric bacteria as antagonists of *Fusarium verticillioides*

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Screening of maize rhizospheric bacteria with antagonistic activity against *Fusarium verticillioides* and plant growth-promoting activities

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Keywords: *Fusarium verticillioides*, antagonists, PGPR, biocontrol microorganisms

ABSTRACT

Aims

This study's aim was to screen bacterial isolates from the maize rhizosphere for use as novel native biocontrol agents against *Fusarium verticillioides* (*Fv*), the main fungal agent of stalk, ear and root rot (SERR) in maize.

Methods

11,520 bacterial isolates were screened for potential activity against *Fv* using a large-scale liquid antagonism assay. Further selection used dual cultures in solid medium, and a hemolytic test to select isolates without human pathogenicity. Antagonistic mechanisms were monitored using plate tests. Finally, potentially antagonistic bacterial isolates were selected for field testing by *in planta* assays.

Results

The liquid antagonism assay selected for 622 isolates that decrease *Fv* growth (≥ 60 %). Dual cultures in solid medium further selected for 42 bacteria inhibiting *Fv* growth (≥ 45 %), representing *Bacillus*, *Pseudomonas* and *Paenibacillus.* Hemolysis assays selected for fourteen isolates. Antagonistic activity analysis revealed that different strains produce glucanases, proteases or chitinases, as well as siderophores and auxins. *In planta* assays demonstrated that three *Bacillus* isolates (*B*5, *B*25 and *B*35) had the highest antagonistic activity against *Fv* (~50 % SERR decrease), while others promoted root volume.

Conclusions

This study enabled us to select for native strains with biocontrol potential against *Fv*. These biocontrol agents are currently being tested in the field, showing effectiveness in reducing the incidence and severity of SERR. Future application of these agents could reduce the use of chemical pesticides and fertilizers.

Abbreviations: *Fusarium verticillioides: Fv*; stalk, ear and root rot of maize: SERR

Introduction

Maize (*Zea mays* L.) is one of the most important cereals grown worldwide. This plant species is also the host of various fungi that can produce mycotoxins in its grains. *Fusarium verticillioides* (Sacc.) Nirenb. (synonym: *F. moniliforme* Sheldon; teleomorph: *Gibberella moniliformis*) is the most commonly reported pathogenic maize fungus that causes stalk, ear and root rot (SERR). This fungus produces toxins with harmful effects to animal and human health (Marschik *et al.*, 2013); it is also responsible for important economic losses worldwide, since its occurrence in maize fields is very high at harvest time (Hernández-Rodríguez *et al.*, 2008b). This has serious implications in Mexico, where maize is one of the most important crops due to cultural consumption habits, economic profitability and extension of culture. Some maize seed batches with a high incidence of *Fv* experience little or no reduction in germination or seedling growth, while the fungus may seriously affect others. In addition to its effects on yield, the infection can be detrimental to grain quality (Munkvold *et al.*, 1997).

Fungal diseases in crops are usually managed by cultural practices, fungicide applications, and the use of resistant maize cultivars. The use of pesticides and fungicides is a controversial practice: it helps increase production, but also has undesirable environmental effects and may lead to increased fungal resistance (Winding *et al.*, 2004). In recent years, the monoculture of maize has caused this crop to become an ideal target for diseases and plagues that rapidly spread, causing a reduction in crop stability (Zavaleta, 1999).

The field of biocontrol for soil-borne plant pathogens was initiated several years ago, and it has become important for its sustainable agronomic practices (Winding *et al.*, 2004). The commercial-scale use of biological agents to control pathogens is a

recent practice, and a number of promising experimental approaches are being developed (Bressan, 2003b). For example, plant growth-promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere (at the rhizoplane or in association with roots) that can improve the extent or quality of plant growth either directly or indirectly (Ahmad *et al.*, 2008). A large array of bacteria, including species from *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligens, Arthobacter, Burkholderia, Bacillus* and *Serratia*, have been reported to enhance plant growth (Podile y Kishore, 2006). The direct promotion by PGPR requires that either the plant is provided with plant growth-promoting substances that are synthesized and secreted by the bacterium, or that the uptake of certain plant nutrients from the environment is facilitated. The indirect promotion of plant growth occurs when PGPR reduces or prevents the deleterious effect of one or more phytopathogens (Ahmad *et al.*, 2008). Achieving the maximum growth-promoting interaction between PGPR and plants relies upon uncovering the mechanisms that rhizobacteria use, and whether they are altered by environmental factors or the interaction with other microorganisms (Bent *et al.*, 2001). Unfortunately, the interaction between PGPR and plants can be unstable, and the results obtained *in vitro* cannot always be dependably reproduced in field conditions (Pereira *et al.*, 2011b).

Coating seeds with biocontrol agents in order to treat them is an appropriate method to suppress plant pathogens in the spermosphere and rhizosphere (Pereira *et al.*, 2007a). In recent years, bacterial inoculants have been used to antagonize soil-borne plant pathogens such as *Fv* and to promote plant growth. *Bacillus subtilis* (Cavaglieri *et al.*, 2005c) and *Pseudomonas cepacia* (Hebbar *et al.*, 1992a) have been used to control *Fv* in maize roots in Argentina and Australia, respectively. *Burkholderia* sp. stimulates plant growth and suppresses disease caused by *Fv* in maize (Hernández-Rodríguez *et*

al., 2008b). *Bacillus amyloliquefaciens* and *Enterobacter hormaechei* reduced *Fv* infection and fumonisin accumulation in maize kernels (Pereira *et al.*, 2010).

Several lines of evidence do not support the use of non-native biocontrol agents. The introduction of a large quantity of "exotic" microorganisms may disrupt a local ecosystem, resulting in unintended ecological impacts to the rhizosphere microbiota (Jackman *et al.*, 1992). Microbial control agents, once released, might not only repress plant pathogens, but also affect non-target microorganisms (Nacamulli *et al.*, 1997). Marked perturbations could arise, eventually leading to permanent alterations in the composition of indigenous microbial populations, and causing nutrient limitations, displacement of indigenous populations and long term suppression of fungal populations in soil (Compant *et al.*, 2005). Due to the limitations of using microorganisms that are not indigenous to a particular soil agroecosystem, current practices focus on designing novel strategies to find native biocontrol agents (Etcheverry *et al.*, 2009) that could regulate *Fv* populations from the same maize fields where they were previously isolated. Moreover, field studies are necessary to acquire an integral view of the interrelationships between plants, pathogens and biocontrol agents, which will help evaluate the safety of introducing biocontrol agents in field trials. The present study focused on screening rhizobacteria isolates that are native to northern Sinaloa, using different selection techniques to find potential *Fv* biocontrol agents for treating at a regional scale. This work should help improve our understanding of the isolates' plant growth-promoting and antagonistic mechanisms.

MATERIALS AND METHODS

Maize rhizobacterial collection

A bacterial culture collection (Scientific collection CIIDIR-003) was used to screen for *Fv* antagonists. This collection is described in Cordero-Ramírez et al. (2013a). Briefly, it consists of 11,520 bacterial isolates taken from five paired groups of plants, each pair consisting of one SERR symptomatic and one asymptomatic plant adjacently sampled from a single row in the field. The field sampling took place in February and March of 2009 in five different locations in northern Sinaloa, Mexico. Microbiological analyses were conducted in each of the five field-sampling points to confirm SERR symptomatology, and *Fv* was isolated from SERR symptomatic plants in selective media.

Fusarium verticillioides

A *Fusarium verticillioides* (*Fv*) isolate was obtained as a monoconidial culture from SERR symptomatic root tissue. This was identified molecularly (Genbank accession no. GU982311.1) and tested for pathogenicity, showing high aggressivity to maize in seedling assays (Figueroa-López *et al.*, 2013).

The experiments used *Fv* conidia suspensions as inoculum. To produce conidia, the fungal isolate was plated in potato dextrose agar (PDA), and incubated at 25 °C for 7 days. Conidia were collected by adding a known volume of sterile water and scraping them off the plate surface using a stainless-steel triangle. *Fv* conidia were counted with a Neubauer chamber to estimate the concentration, and this was adjusted to 2×10^5 conidia/mL for experiments.

Large-scale liquid antagonism screening assay

This protocol was performed using 11,520 frozen bacterial stocks from Scientific collection CIIDIR-003, as described in Figueroa-López et al. (2013). The criterion used to select a good antagonist was arbitrarily set at ≥60 % fungal growth inhibition.

Dual culture antagonism assays in 96-well plates using solid medium

Bacterial isolates derived from the liquid antagonism screen were assayed as described in Figueroa-López et al. (2013). Inhibition percentage was calculated as (halo mm / maximum inhibition mm) x 100, in which the diameter of the well is the maximum inhibition and the halo is the radius of the fungus (in mm), due to bacterial isolate inhibition. In this assay, the selection criterion was set for isolates to display ≥45 % antagonism in all three replicates.

Blood hemolytic assay

Hemolysis tests were performed in order to discard isolates that could be pathogenic for humans, and to avoid working with isolates that produce complete β-hemolysis in this test. Bacterial isolates were grown in 15-mL tubes containing 5 mL of Luria Bertani (LB) medium at 25 °C for 24 h, at 250 rpm. One mL of bacterial culture was taken and transferred to a 1.5 mL tube, centrifuged twice at 16,800 g for 5 min and the resulting supernatant was transferred to a new tube. 5 mm-diameter wells were made in blood agar plates with a cork borer, and 50 µL of supernatant were placed in the wells. The plates were stored at 37 °C for 24 h. Complete β-hemolysis was observed as a clear zone around the well in the blood agar medium, indicating complete breakage of

erythrocytes. On the other hand, partial α-hemolysis was observed as a dark-green coloration around the well, indicating the partial damage of erythrocytes. Bacteria with γ-hemolysis do not exhibit any alteration of color or opacity in the medium, indicating an absence of hemolysis (Forbes *et al.*, 2002). Partial α-hemolysis of the isolates was very small, and it was not easy to conclude they were hemolytic even after repeating the assays three times and in triplicate. We therefore chose to continue working with those isolates that showed partial or no hemolysis.

In planta antagonism assay

Two types of white maize hybrid seeds (Cebú and Garañón, both from Asgrow) were used for the *in planta* antagonism assay. Seeds were surface-sterilized prior to bioassays by placing them in 0.75% sodium hypochlorite at 52 °C for 20 minutes, followed by three abundant washes with sterile distilled water for five minutes each. Subsequently, seeds were pre-germinated on Komada's *Fusarium*-selective medium (Komada, 1975), and seeds with no symptoms of fungal growth (*i.e.*, *Fv* free) were selected. Bacterial isolates were grown in 15-mL tubes containing 5 mL of LB medium at 25 °C for 24 h at 250 rpm, and an optical density (OD) of 595 nm was used to calculate the colony-forming units (CFU/mL) after plating. Maize seeds were soaked in bacterial suspensions containing 1.5 x 10^8 CFU/mL for 20 min. Three seeds were planted per sterile polypropylene container (similar to a Magenta box) containing 200 g of wet sterile sand. An absolute control was included, containing seeds treated with sterile distilled water in sterile sand. An additional control consisted of soaking the seeds with the bacterial isolates and seeding them on wet sterile sand. Treatments were set in sand inoculated with *Fv* two days before sowing. *Fv* was added at a concentration of 1 x 10^5

conidia/g of sand. Nine plants per control or treatment were evaluated in three experimental sets containing three seeds each. The experiment was evaluated 45 days after emergence of the seeds. Root volume was measured (Burdett, 1979) and disease severity was evaluated as described in Cumagun (2009). A completely randomized experimental design was used. The obtained severity scale (Cumagun 2009) values were evaluated by a normality test, using the Shapiro Will test and a Bartlett's test to confirm variance homogeneity. Data were parametric, and severity scale data were subjected to statistical analysis of variance (Chaffin *et al.*) to detect differences between treatments, which were considered significant at $P \leq 0.05$ for mean comparisons. Analyses were performed using the Statistical Analysis System 9.0 software (SAS Institute, Cary, NC).

Characterization of functional plant growth-promoting and/or antagonistic traits

Plate screening assays were used to investigate possible mechanisms that α- or γ-hemolytic bacterial antagonistic isolates use to inhibit *Fv* growth. Plant growth-promoting traits examined include indole-3-acetic acid (IAA) secretion and phosphate solubilization, whereas antagonistic traits include chitinase, glucanase and protease activities, and siderophore production.

Auxin production

To evaluate IAA production, single colonies were grown in LB broth for 24 h at 25 °C, and the supernatants were treated with Salkowsky's reagent (Loper y Schroth, 1986). IAA detection was visualized as the development of pink color, after incubation for 30 min at 25 °C in the dark. The IAA concentration for each sample was estimated

spectrophotometrically (530 nm), by comparison to an IAA (Cat. I2886, Sigma, St Louis, MO, USA) standard curve (0 to 100 µM).

Phosphate solubilization

All isolates were screened for phosphate-solubilizing ability on Pikovskaya's agar (Pikosvkaya, 1948). The bacteria were streaked on Pikovskaya's agar plates and incubated for one week at 25 °C. The presence of a clear zone around the bacterial colony was considered positive.

Chitinase plate assay

Colloidal chitin was prepared from chitin flakes (Cat C-7170, Sigma Chemicals Company St. Louis, MO, USA), according to Shanmugaiah (2008). The chitin flakes were ground to a fine powder, 4 g of which were slowly added to 100 mL 10 N HCl and maintained overnight at 4 °C with vigorous stirring. Cold absolute ethanol (200 mL) was added to the suspension with rapid stirring and maintained overnight at 25 °C. A precipitate was collected by centrifugation at 75,465 g (Beckman JA 25.50 rotor) for 20 min in a Beckman J-30I centrifuge and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). The pellet was freeze-dried and stored at 4 °C until further use. The colloidal chitin agar medium was prepared by mixing 0.5% (w/v) of colloidal chitin with 20 g of agar in minimal medium containing the following components: 5 g/L $(NH_4)_2SO_4$, 1 g/L K_2HPO_4, 10 g/L NaCl, 0.1 g/L $MgSO_4 \cdot 7H_2O$, and 0.5 g/L yeast extract. The medium was subsequently sterilized at 121 ºC and 15 PSI for 20 min. The chitinase assay was performed on colloidal chitin agar medium, and

chitinase activity was identified by the formation of a clear zone around the bacterial cells after 5 days of growth at 25 °C. Each tested isolate was assayed in triplicate.

Glucanase plate assay

Single colonies were grown in LB broth for 48 h at 30 ºC and the cell-free supernatants were tested for *in vitro* β-1, 4-endoglucanase activity using carboxy-methyl cellulose (CMC; Cat 419273, Sigma Chemicals Company St. Louis, MO, USA) as the substrate. Two hundred µL of the supernatant were placed in 5 mm-diameter wells (previously made using a cork borer) in 1% CMC agar plates, and incubated for 24 h at 30 ºC. The formation of a clear zone around a well, resulting from β-1,4-endoglucanase activity, was revealed by adding 5 mL of Congo red 1% w/v for 15 min, then the Congo red dye was removed and 5 mL NaCl 2 M was added for 15 min to eliminate the excess dye, and to visualize the formation of clear zones (Teather y Wood, 1982).

Siderophore production

Siderophore production was determined after one week of incubation in chrome azurol S (CAS) agar. The CAS blue solution for this assay was prepared according to Schwyn and Neilands (1987). Pure isolates were pricked onto CAS agar plates using sterile toothpicks and incubated at 25 °C for 2 weeks in the dark, and the assay was performed in triplicate. The colonies with yellow/orange zones were considered to be siderophore–producing strains. The CAS-agar (non-inoculated) control plates were incubated under the same conditions as described above; no color change was observed following incubation.

Protease plate assay

Protease activity was tested in skimmed milk agar (SMA) with commercially available non-fat milk, according to (Jones *et al.*, 2007). The strains were streaked onto SMA, and the plates were incubated for 24 h at 30 °C. The protease activity was identified by the formation of a clear zone around the bacterial colonies.

RESULTS

Large-scale liquid antagonism assay

11,520 bacterial isolates were screened using a high-throughput liquid assay method (Figueroa-López *et al.*, 2013) (Figure 1A), and 622 isolates showing 60-95 % *Fv* growth inhibition were selected (Table S1). The bacterial isolates from the collection CIIDIR-003 were previously identified using 16S rDNA sequencing (Cordero-Ramírez *et al.*, 2013a), which enabled identifying bacterial genera that display antagonistic activity in this liquid assay. Genera exhibiting antagonistic potential against Fv were: *Bacillus* (53.53 %); *Enterobacter* (6.11 %); *Pseudomonas* (3.70 %); *Lysinibacillus* (2.10 %); *Acinetobacter*, *Paenibacillus* and *Stenotrophomonas* (0.64 % each); *Agrobacterium*, *Arthrobacter* and *Pantoea* (0.48 % each); *Klebsiella* (0.32 %); *Anaerobranca* and *Aquaspirillum* (0.16 % each); and 190 non-identified isolates representing 30.55 % of the total (Figure 2). These 622 isolates were rearranged in a new 96-well plate collection (designated as a *Fusarium* antagonists collection) and cryopreserved at -70 °C to facilitate manipulation.

The most abundant genus, *Bacillus*, caused a reduction in fungal growth from 53 to 99 %, and contained isolates from 13 different species including *B. aquimaris*, *B. arbutinivorans*, *B. aryabhattai*, *B. badius*, *B. cereus*, *B. flexus*, *B. fusiformis*, *B. licheniformis*, *B. marisflavi*, *B. megaterium*, *B. pumilus*, *B. subtilis* and *B. thuringiensis*. The second most abundant genus, *Enterobacter*, included *E. asburiae*, *E. cloacae*, *E. hormaechei* and *E. ludwigii*, which promoted between 64 – 93 % *Fv* growth. The antagonistic *Pseudomonas* species *P. putida*, *P. lini*, *P. corrugata*, *P. stutzeri*, *P. chlororaphis*, *P. pseudoalcaligenes* and *P. fluorescens* identified in this assay exhibited 60 – 93 % *Fv* growth inhibition. The *Lysinibacillus* species *L. sphaericus* and *L. fusiformis* inhibited 62 – 82 % of fungal growth. The *Acinetobacter* species *A. rhizosphaerae*, *A. calcoaceticus* and *A. lwoffii* provoked 81 – 92 % *Fv* inhibition. Additional species such as *Peanibacillus polymyxa*, *Arthrobacter globiformis*, *Pantoea dispersa*, *Anaerobrancha californiensis* and two species of *Klebsiella* showed *Fv* growth inhibition in this assay (Table S1).

In vitro dual culture solid assay in 96-well plates

The selection process included a second test that followed the same principle of a conventional dual culture in solid medium in Petri dishes, except that it was carried out in 96-well plates. This assay was used to determine the *in vitro* antagonistic activity observed in the liquid bioassay (Figure 1B). Forty-two out of the 622 selected bacterial isolates from the *Fusarium* antagonists collection exhibited 45-85% *Fv* growth inhibition, and are listed in Table 1. Most isolates belonged to the genus *Bacillus* (34 isolates), which was represented by *B. cereus* (15 isolates), *B. thuringiensis* (6 isolates), *B. megaterium* (6 isolates), and *B. subtilis* (5 isolates), *Bacillus* sp. (1 isolate) and

Bacillus *flexus* (1 isolate). One isolate from each of *Pseudomonas putida*, *P. fluorescens* and *Paenibacillus polymixa* also inhibited *Fv* growth *in vitro*, as well as five undetermined isolates.

Hemolytic test

This assay was performed to discard isolates with a possible pathogenic effect in humans, as judged by their ability to produce hemolysins. Six isolates showed partial or α-hemolysis (*B*2, *Ps*3, *B*5, *B*7, *B*12 and *B*13), and eight isolates were γ-hemolytic (*B*4, *Pa*8, *B*9, *B*22, *B*23, *B*24, *B*25 and *B*35). The 28 bacterial isolates exhibiting total or β-hemolysis were discarded (Table 1). The remaining isolates were used in the following *in planta* selection step, to continue testing for their antagonistic behavior in the presence of the host plant (Figure 1C).

In planta assays

The maize seeds used in this experiment were cleaned according to Daniels (1983). Using this methodology, we obtained 98-100% seed germination and 1-3% seed contamination. For this reason, seeds were pre-germinated and those that presented contamination were eliminated. Only *Fusarium*-free seeds were used for the *in planta* assay (Figure 1D). The criterion for hybrid selection was based on those hybrids that are most extensively used in the maize fields of Guasave, Sinaloa. Cebú was first used during the selection of antagonists in the lab while Garañón is now preferred after several consecutive crop cycles of the previous Cebú hybrid. Currently the most extensively used hybrid in this area is Gorilla.

One isolate out of 13 showed a significant difference in root volume: the *B*13 isolate increased about 60% in root volume as compared to the control inoculated with *Fv*. All other isolates tested in the Cebú hybrid did not show any significant differences in root volume (Figure 3A). Regarding the percentage of *Fv* disease severity in this hybrid, isolates *B*5 (47%), *B*13 (62%) and *B*25 (53%) significantly reduced *Fv* disease severity as compared to the control (100%) (Figure 3B).

In the Garañón hybrid, isolates *Ps*3, *B*5, *B*25 and *B*35 showed significant increases in root volume, in comparison to the control inoculated with *Fv* (Figure 3C). The percentage of *Fv* severity disease was reduced 45 to 50% by *B*5, *B*25 and *B*35 isolates, as compared to the *Fv*-treated control (Figure 3D).

The isolates *B*5 and *B*25 displayed a similar effect in disease severity, reducing the level of SERR caused by *Fv* in both maize hybrids. The *B*35 isolate reduced *Fv* disease severity in Garañón but not in Cebú, and *B*13 was active in Cebú but not in Garañón, suggesting that the *Fv* antagonistic activity of these isolates is hybrid-specific (Figures 1B and 1D).

Plant growth-promoting and antagonistic traits of bacterial isolates tested *in planta*

The isolates tested in maize antagonistic assays were analyzed to elucidate the possible mechanisms responsible for *Fusarium* biocontrol. Screening results from the plant growth-promotion test are illustrated in Table 2. Phosphate solubilization was detected in the *B*4, *B*5, *Pa*8, *B*12, *B*13 and *B*23 isolates. IAA production was only observed for the *Pa*8 isolate, which produced 40 µM of auxin-like compounds. The isolates *B*13, *B*23, *B*24 and *B*25 exhibited chitinase activity. Production of siderophores was observed in *Ps*3, *B*4, *B*5, *B*7, *B*12, *B*13, *B*22, *B*24 and *B*25. Protease activity was present in *B*4,

*B*5, *B*7, *B*12, *B*22, *B*24 and *B*25. All isolates had glucanase activity except for *B*12 and *B*23. Isolate *B*25 was chosen for the purpose of illustrating plate results, as it possesses multiple enzymatic activities. These include chitinase and protease (observed as clear zones around the colonies; Figures 4A and 4B), and glucanase (observed as a clear halo around the wells containing the bacterial supernatant; Figure 4C). Siderophore production was observed in Chrome azurol S agar as a color transition from blue to orange/yellow, adjacent to bacterial colonies (Figure 4D).

Discussion

One of the main reported diseases that affects maize is SERR (Vigier *et al.*, 2001). This study was carried out to identify native rhizospheric bacteria able to control *Fv*. For this, we used a bacterial collection containing 11,520 native isolates from the rhizosphere of maize plants taken from maize fields located in the Guasave Valley of Sinaloa, Mexico. Manipulating large amounts of microorganisms can be difficult, and thus we previously developed a high-throughput methodology using a liquid antagonistic assay (Figueroa-López *et al.*, 2013). Here, we used this new methodology to screen for potential antagonists from this microorganism collection. The basis for our novel technique is wheat germ agglutinin (WGA), a lectin which recognizes the N-acetyl-D-glucosamine and N-acetyl-D-neuraminic (sialic) acid residues from chitin found in the fungal cell wall. This lectin is conjugated to Alexa Fluor 488 (WGA, Alexa Fluor® 488 conjugate), a fluorophore that allows estimating fungal biomass and fungal growth inhibition by comparing treatments to a fungus-only control. Possible mechanisms of growth inhibition in such a liquid assay where the bacteria and the fungus are in contact with

each other include direct mycoparasitism (Manjula *et al.*, 2004), and fungal cell wall degradation by the action of enzymes such as chitinases (Liu *et al.*, 2011), glucanases (Liu y Du, 2012), or proteases (Chang *et al.*, 2008). Antibiosis could also be enabled, by producing compounds that halt pathogen growth such as siderophores (Yu *et al.*, 2011), or antibiotics such as the antifungal lipopeptides zwittermicin A (Silo-Suh *et al.*, 1994) and kanozamine (Milner *et al.*, 1996). Both siderophores and kanozamine, may act by affecting spore germination and/or hyphal elongation.

The large-scale liquid antagonism screening assay enabled identifying isolates that are potential *Fv* antagonists. More than half of the *Fv* antagonistic isolates were represented by different *Bacillus* species (Table S1). Consistent with this, *Bacillus* species have been reported to produce antibiotics and to use diverse mechanisms that may inhibit this fungal pathogen (Ongena y Jacques, 2008). These mechanisms include: i) nutrient competition, as fungi in the environment require exogenous nutrients like carbon and iron to germinate, penetrate and infect (Kamilova *et al.*, 2005); ii) production of antifungal lipopeptides (Nihorimbere *et al.*, 2012); iii) inhibition of spore germination by different compounds (Chandel *et al.*, 2010); and iv) production of lytic enzymes that degrade the fungal cell wall components (Liu *et al.*, 2011). *Bacillus* can produce chitinases to degrade the fungal cell wall, to prevent hyphal extension (Kishore *et al.*, 2005).

Pseudomonas was the third most abundant genus exhibiting *Fv* antagonistic activity in our liquid assay. This genus has been extensively studied, and its congeners have been reported to synthesize different lytic enzymes, antibiotics, cyanide, salicyclic acid and siderophores, and even to solubilize phosphate (Nagarajkumar *et al.*, 2004). In this study, *Pseudomonas putida* and *P. fluorescens* exhibited *Fv* growth inhibition in the

solid medium assay (Table 1). *Pseudomonas* species can produce β-1-3 glucanase, and affect diverse fungal plant pathogens (Gorlach-Lira y Stefaniak, 2009).

The antagonistic activity detected in the large-scale liquid antagonism assay was verified by a confirmatory assay in solid medium, implemented in 96-well plates (Figueroa-López *et al.*, 2013). In this work, we noted *Fv* growth inhibition from 60 to 95% in liquid medium assays, and from 45 to 85% in solid medium assays. The main antagonistic mechanism observed in dual cultures on solid medium is antibiosis (Shali *et al.*, 2010), although nutrient sequestration or competition are other mechanisms that can be assessed through these types of assays (Taurian *et al.*, 2010). Most of the 42 isolates selected in the solid assay belong to the genus *Bacillus* (Table 1). There are many reports about the biocontrol activity of *Bacillus* species against plant pathogens (Chang *et al.*, 2008). Cavaglieri et al. (2005a) demonstrated the antagonistic effect of ten *Bacillus* isolates against *Fv*, with growth inhibition ranging from 28 to 78%. Chang et al. (2008) reported the production of antifungal proteases produced by *B. cereus*. In a more recent study, the production of chitinases by *Bacillus* was demonstrated to reduce *F. graminearum* infection in wheat (Shali *et al.*, 2010).

Paenibacillus polymyxa was also identified as an antagonist in solid medium against *Fv* in this study. This species promotes plant growth by producing cytokinins, auxins, ethylene, and by fixing nitrogen (Heulin *et al.*, 1994). It can also solubilize phosphate (Singh y Singh, 1993), and produce antibiotics (Beatty y Jensen, 2002), and hydrolytic enzymes (Yang *et al.*, 2004). These traits could be responsible for its antagonistic effect against *Fv* (Table 1). In addition, this strain can reduce the mycelial growth of *F. oxysporum, Phytopthora palmivora* and *P. aphanidermatum* (Lal y Tabacchioni, 2009). This species also produces fusaricidin, an antifungal compound that works against various plant pathogens such as *F. oxysporum, Aspergillus niger, A.*

oryzae and *Penicillium thomii* (Kajimura y Kaneda, 1996). Similarly, *Pseudomonas putida* and *P. fluorescens* exhibited *Fv* growth inhibition in the solid medium assay. *Pseudomonas* species have been reported to affect a number of fungal phytopathogens such as *F. oxysporum, Rhizoctonia solani, Sclerotium rolfsii* and *Pythium ultimum* (Gorlach-Lira y Stefaniak, 2009).

When working with microorganisms in a crop that will be consumed by humans, care must be taken to avoid those which may exhibit human pathogenicity. Hemolysis is a standard test for possible human pathogens, and we monitored hemolytic activity in order to remove isolates that clearly showed complete hemolytic activity. The partial hemolytic isolates showed very weak activity, and it was thus difficult to conclude if they were hemolytic or not. We therefore selected those isolates showing partial or no hemolysis for *in planta* assays (Table 1). Avoiding potential human pathogens in biological plant protection requires evaluating the risk of each biological control agent (Berg, 2009; Cordero-Ramírez *et al.*, 2013b). Although an important feature displayed by human pathogens is blood hemolysis, other tests are necessary to complement hemolysis tests, as proposed by Zachow et al. (2009).

Once potential antagonistic isolates are obtained *in vitro* by challenging bacteria against *Fv*, it is important to consider the plant host when performing biocontrol assays. Several reports indicate that *in vitro* results do not work as expected in assays with a bacteria-fungus-plant association (Szczech y Shoda, 2006).

This decreased performance is probably due to the exchange of chemicals in plant exudates, which could affect the fungus or bacteria in different ways (Fan *et al.*, 2012). Furthermore, the expected results may differ when a potential bacterial antagonist is applied in field conditions, and other biotic (accompanying microbiota) or abiotic factors (physicochemical conditions of the soil, climate, water availability) are

taken into account(Egamberdiyeva, 2007). Sometimes, biocontrol agents do not exhibit plant promoting effects in field trials with maize plants, thus indicating that they may not act as biofertilizers (Pereira *et al.*, 2011b). On the other hand, there were satisfactory results using an endophytic strain of *Bacillus subtilis* in maize, which resulted in reduced mycotoxin production and decreased *Fusarium verticillioides* colonization (2001).

Application of bacteria to seeds has been widely used for the biological control of soil-borne plant pathogens that affect many host plants, and some studies report using seed bacterization to control *Fv* in field assays (Hebbar *et al.*, 1992c; Cavaglieri *et al.*, 2005a). In this work we found 14 native isolates of the maize rhizosphere that are able to inhibit the growth of *Fv in vitro* (Table 2), which were tested by application to maize seeds in lab experiments. *Pseudomonas putida* (*Ps*3), *Bacillus* spp. (*B*5, *B*35) and *B. cereus* (*B*25) increased root volume in the white maize hybrid Garañón, while *B. subtilis* (*B*13) increased root volume in Cebú (Figure 3). *Bacillus subtilis* has been reported to increase seed germination and root and shoot length in maize, and was effective against *F. oxysporum* in reducing stalk wilt in tomato plants (Omar *et al.*, 2006). *Bacillus megaterium* (*B*5) and *B. cereus* (*B*25) reduced *Fv* disease severity in both white maize hybrids tested. *Bacillus megaterium* (*B*5) was revealed to be a good potential biocontrol agent in the plant antagonistic assay (Figure 3). Similar results have been reported in *Fusarium* crown and root rot of tomato (Omar *et al.*, 2006). *Bacillus cereus* (*B*25) reduced *Fv* disease severity in two white maize hybrids tested in this study; these results are similar to those found by Cavaglieri et al. (2005a), who demonstrated the antagonistic effect of different *Bacillus* isolates on *Fv* growth inhibition. Many secondary metabolites are produced throughout the *Bacillus* genus, which have an antifungal effect on diverse plant pathogens (Raaijmakers y Mazzola, 2012). Here, we

performed several tests to investigate the possible mechanisms that the isolates use to cause *Fv* growth inhibition and decrease disease severity in maize plants, such as enzymatic activity and siderophore production.

Phosphate-solubilizing activity is determined by the microbial biochemical ability to produce and release organic acids. Through their carboxylic groups, the organic acids can chelate the cations (mainly Ca^{2+}) bound to phosphate, converting them into the soluble forms (Kpomblekou-a y Tabatabai, 1994). Conversely, soluble forms can be obtained through the production of phosphatases that solubilize phosphorus insoluble forms (Richardson *et al.*, 2009). Phosphate-solubilizing bacteria can play an important role in plant nutrition by increasing phosphorus uptake in plants (Rodríguez *et al.*, 2007). The isolates that solubilized tricalcium phosphate *in vitro* were *Bacillus* spp. (*B*4, *B*5, *B*23), *B. subtilis* (*B*12), *B. subtilis* (*B*13) and *P. polymyxa* (*Pa*8) (Table 2). This suggests that these isolates could be beneficial for phosphate nutrition and growth in maize.

Furthermore, *Paenibacillus polymyxa* (*Pa*8) was the only isolate that produced indol-acetic acid *in vitro* (Table 2), although it did not increase plant root volume (Figure 3). This species has been previously reported to produce different phytohormone-like compounds such as auxins (Acuña *et al.*, 2011), cytokinins and ethylene, and to protect the plant against fungal pathogens (Lal y Tabacchioni, 2009). The fact that it did not produce any effect in the plant roots may be due to a multitude of different factors that affect auxin production or its transport from the rhizosphere to the root system (Woodward, 2005).

In our study, the isolates *B. cereus* (*B*24 and *B*25), *B. subtilis* (*B*13) and *Bacillus megaterium* (*B*23) showed chitinase activity in colloidal chitin agar (Table 2). This

suggests that chitinolytic activity may be a potential control mechanism for these isolates.

The siderophores produced by PGPR can inhibit root pathogens by creating limiting iron conditions in the rhizosphere. Yu et al. (2011) reported a *Bacillus subtilis* strain that produces bacilibactin and itoic acid as well as siderophores, and is able to induce systemic resistance to *Fusarium* wilt in pepper. According to our findings, the *Bacillus* isolates *B*5, *B*13 and *B*25 are siderophore producers and potential biocontrol agents that can reduce the disease severity in maize plants (Table 2 and Figure 2). Isolates with auxin or siderophore production, or phosphate-solubilizing activity, could be used to enhance plant growth in combination with compatible isolates. This could offer a higher *Fv* antagonist potential *in planta*, since applying it as a bacterial consortium could result in both: 1) plant growth promotion and better nutrient acquisition; and 2) protection against SERR in maize.

Bacillus spp. use a diverse arsenal of antifungal and antimicrobial metabolites that could potentially be used as control agents (Chang *et al.*, 2008). Several studies indicate that *Bacillus* spp. excrete antifungal proteins, causing inhibition of *F. oxysporum*, *F. solani*, *P. ultimum* and *Rhizoctonia solani* (Chang *et al.*, 2008; Gao *et al.*, 2008). In this work, we tested the ability of *Bacillus* isolates to produce diverse enzymes such as proteases, which are considered key players in the cell wall lysis of higher fungi. *Bacillus* isolates *B*5 and *B*25 produce proteases, and this enzymatic activity could possibly act by lysing fungal-secreted hydrolytic enzymes or by damaging *Fv* cell walls/membranes and causing a reduction in SERR disease severity (Table 2).

Several other enzymatic activities were also examined, revealing that all isolates produce glucanases except for *B*12 and *B*23 (Table 2). Chitin and glucan are the main

structural components of the fungal cell wall. Therefore, the chitinases and glucanases excreted by antagonists have been suggested to be the key enzymes in the cell wall lysis of soil-borne phytopathogenic fungi during mycoparasitic action (Yang *et al.*, 2004).

Altogether, our results suggest that the isolates *B. megaterium B*5 and *B. cereus B*25 have the most promise as potential *Fv* control agents. They increased root volume in one of the two tested maize hybrids, and they were able to diminish *Fv* disease incidence in both hybrids. Isolate *B*5 is able to solubilize phosphate, whereas *B*25 has chitinase activity; both are able to produce siderophores, and have protease and glucanase activities. These two isolates (*B*5 and *B*25) and *Bacillus* sp. (*B*35), an isolate that only produces glucanases but which can induce root volume and effectively decrease disease incidence in Garañón, are currently being tested in maize fields sown with the Garañón white maize hybrid in northern Sinaloa (Table 2 and Figure 3).

Intriguingly, some bacteria may share similar repertoires of hydrolytic enzymes or antagonistic traits when tested *in vitro*, but the results may differ when the plant and the fungus are taken into account in this tripartite interaction. This is suggested by the fact that *B*4 and *B*5, or *B*24 and *B*25, share similar activities, although no *Fv* control was exerted by *B*4 or *B*24. The reasons for this are currently not understood, but parallel studies to elucidate the mechanisms that these bacteria use to exert biocontrol in plants are currently being explored.

Studies have been performed in our group and parallel to this work that use the same microorganism collection. The aim of these studies is to characterize the cultivable maize rhizosphere bacterial populations from this collection that are related to SERR. Accordingly, our results revealed that the two *Bacillus* isolates *B*5 and *B*25 could be assigned to Operational Taxonomic Units (OTU) 6 and 2 respectively. These two OTUs are highly represented (1,556 and 985 out of 7,077 16S rDNA sequences,

respectively). Furthermore, the *B. cereus B2*5 isolate belonging to OTU 2 is affected by the infection status, with a higher representation in SERR symptomatic plants. It is possible to envision that community changes in the rhizosphere of maize plants affected by SERR result in an increase in bacterial populations that are antagonistic to *Fv*, in an attempt to control the *Fv* infection.

Field studies using these isolates are now essential to corroborate our findings from *in vitro* and *in planta Fv* antagonistic assays. Succesful implementation of these results in the future will improve the application of these isolates as biocontrol agents, which will also reduce the use of chemical pesticides and fertilizers in the large extensions of maize fields in Sinaloa, Mexico and similar regions worldwide.

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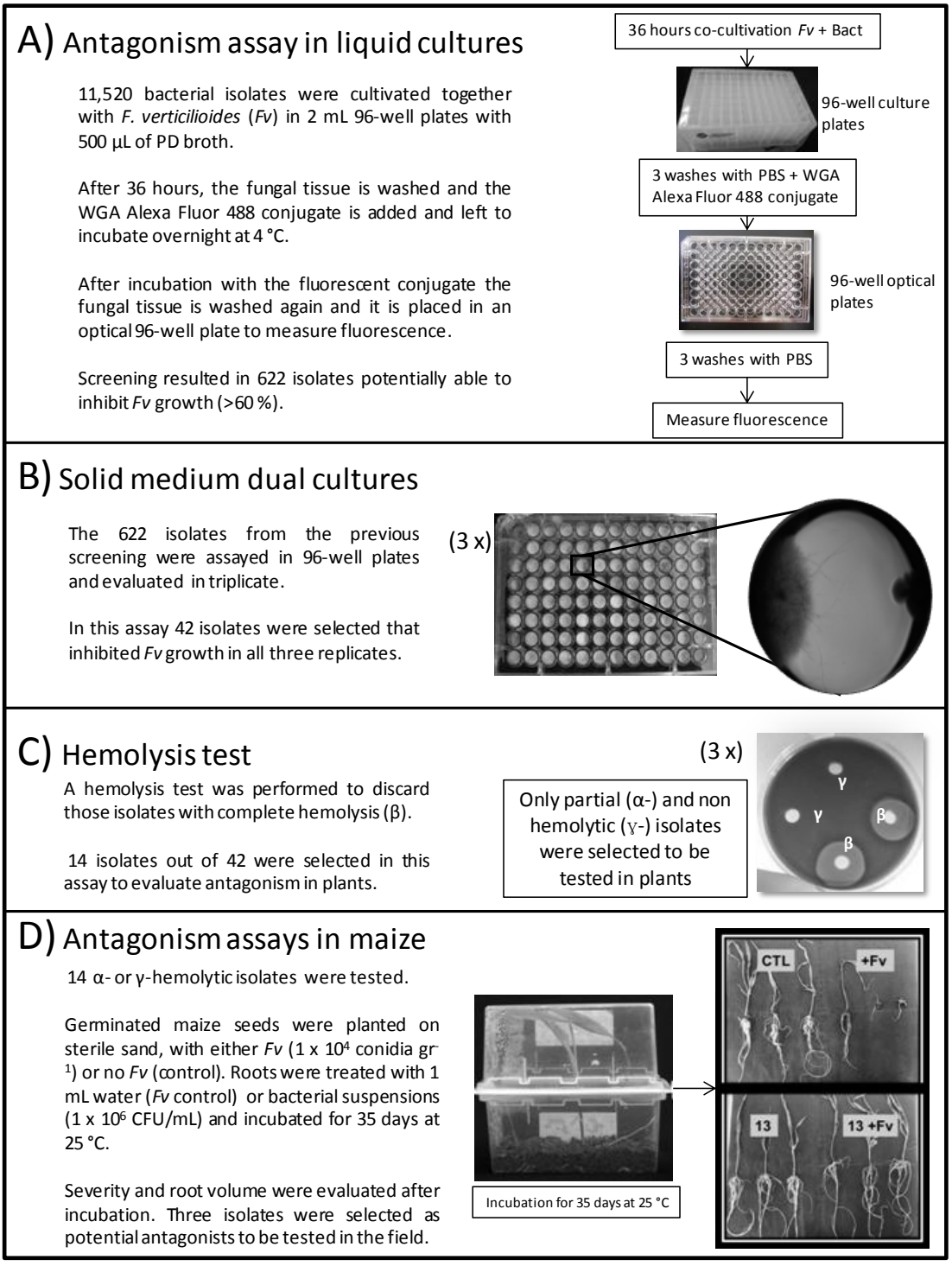

Fig. 1 Scheme showing the experimental design protocol for selection of bacterial antagonists against *Fv* from the maize rhizosphere.

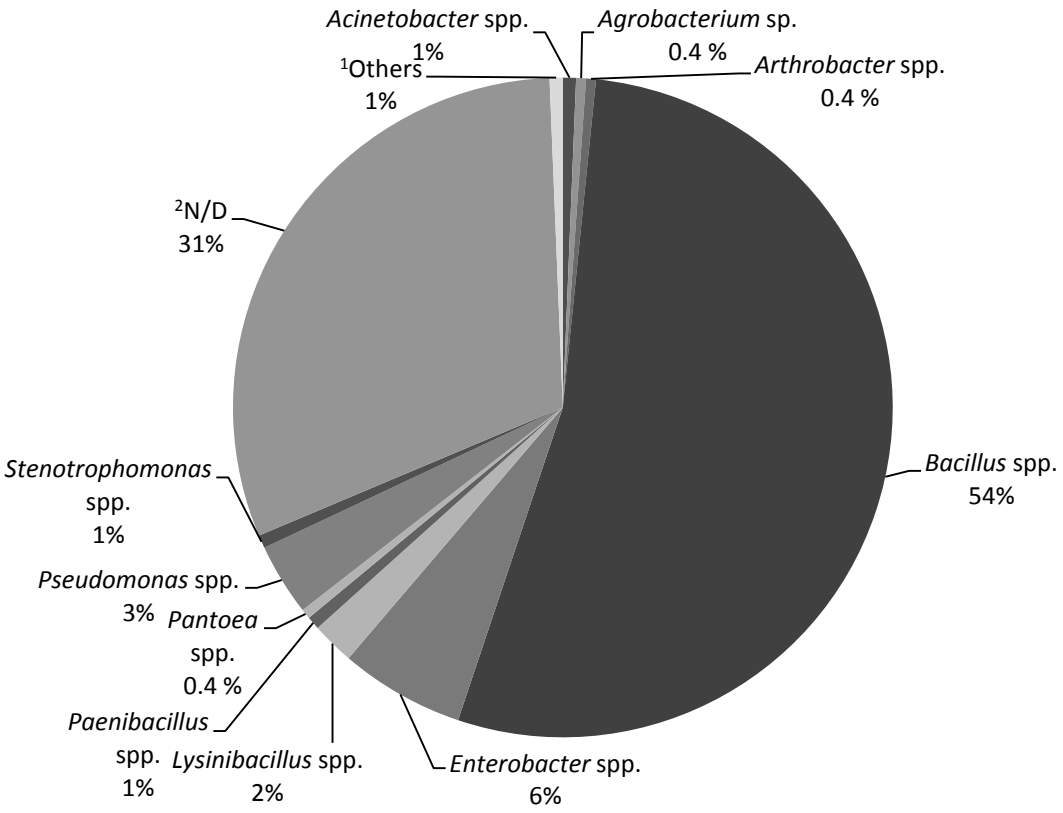

Fig. 2 Main bacterial genera representing the 622 isolates with a potential antagonistic effect against *Fv*, after the initial screening in liquid cultures. Genera are reported as the percentage of the 622 isolates.

[1]Others include the genera *Anaerobranca* sp. (one isolate); *Aquaspirillum* sp. (one isolate) and *Klebsiella* sp. (two isolates).

[2]N/D stands for not determined.

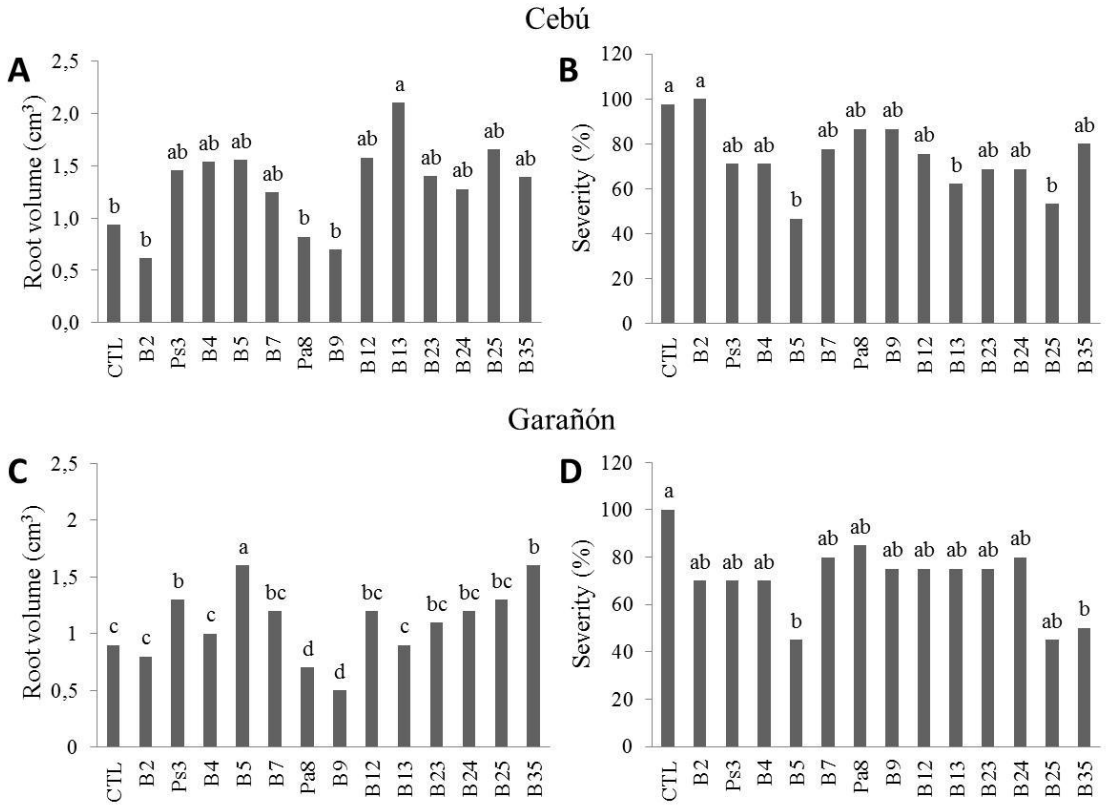

Fig. 3 *In planta* antagonistic assays in two white maize hybrids inoculated with 14 partial or non-hemolytic bacterial isolates and *Fv*, 45 days after seed emergence. A) Root volume (Cebú hybrid), B) Percentage of disease severity (Cebú hybrid), C) Root volume (Garañón hybrid), D) Percentage of disease severity (Garañón hybrid). CTL refers to the fungus control (plant plus *Fv)*. Letters preceding a number indicate the genus of that particular isolate: *B* refers to *Bacillus*, *Ps* is *Pseudomonas* and *Pa* is *Paenibacillus*. Identical letters appearing above bars indicate no significant differences, while different letters indicate significant differences (Tukey p ≥0.05). *B*22 was only tested in the Garañón maize hybrid.

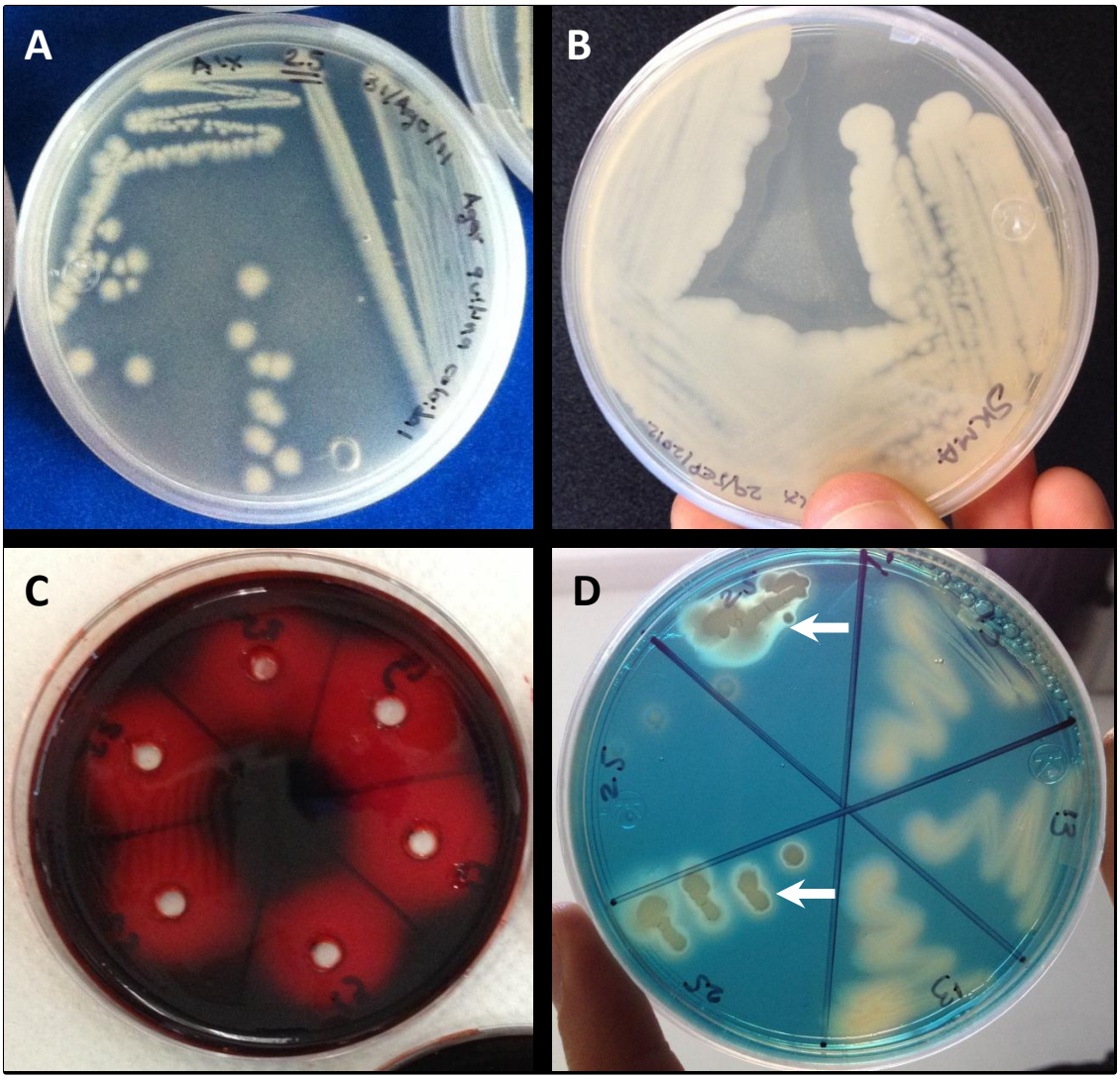

Fig. 4 Antagonistic traits of *B*25 isolate. A) Chitinase assay performed in colloidal chitin agar plates, five days after bacterial inoculation and incubation at 25 °C. The clear zone surrounding the colonies indicates chitinase activity. B) Protease assay performed in SMA plates, 24 hours after inoculation and growth at 30 °C. A clear zone around the bacterial colonies indicates protease activity. C) Glucanase assay, performed by adding bacterial supernatant (after 48 h of growth at 30 °C) to 5-mm diameter wells made in CMC agar plates. After inoculation and 24 h of incubation at 30 °C, Congo red dye (1 % w/v) was added for 15 min to stain CMC. The dye was then removed to reveal the formation of clear

zones around the wells, which are considered a positive result for glucanase activity. D) Siderophore production was observed by pricking bacterial inoculum in CAS agar plates and allowing bacteria to grow for 2 weeks at 25 °C. The clear zones surrounding bacterial colonies around *B*25 (white arrows) indicate siderophore production, whereas *B*13 did not show any siderophore production.

Table 1 Percentage of *Fv* growth inhibition in the liquid medium (PDB) and solid medium assays (PDA), as well as type of hemolysis, for the 42 isolates selected in the solid antagonistic assay yielding ≥45% *Fv* growth inhibition.

Name	Isolates	% inhibition in PDB	% inhibition in PDA	Hemolytic type
[1]***B*1**	*Bacillus megaterium*	87	60	β
***B*2**	*Bacillus megaterium*	88	66	α
***Ps*3**	*Pseudomonas putida*	84	67	α
***B*4**	*Bacillus flexus*	89	71	γ
***B*5**	*Bacillus megaterium*	91	66	α
***B*6**	*Bacillus subtilis*	66	73	β
***B*7**	*Bacillus megaterium*	74	71	α
***Pa*8**	*Paenibacillus polymyxa*	62	85	γ
***B*9**	*Bacillus cereus*	62	70	γ
***B*10**	*Bacillus cereus*	83	57	β
N11	[2]N/D	84	56	β
***B*12**	*Bacillus subtilis*	79	49	α
***B*13**	*Bacillus subtilis*	86	63	α
***B*14**	*Bacillus cereus*	81	62	β
***B*15**	*Bacillus cereus*	80	63	β
***B*16**	*Bacillus subtilis*	81	69	β
***B*17**	*Bacillus subtilis*	82	67	β
***B*18**	*Bacillus thuringiensis*	85	82	β
***B*19**	*Bacillus thuringiensis*	71	72	β
***B*20**	*Bacillus thuringiensis*	85	72	β
***B*21**	*Bacillus thuringiensis*	82	64	β
***B*22**	*Bacillus megaterium*	74	68	γ
***B*23**	*Bacillus megaterium*	64	63	γ
***B*24**	*Bacillus cereus*	72	73	γ
***B*25**	*Bacillus cereus*	93	52	γ
***B*26**	*Bacillus cereus*	74	58	β
N27	N/D	75	47	β
***B*28**	*Bacillus cereus*	79	52	β
***B*29**	*Bacillus thuringiensis*	72	63	β
***B*30**	*Bacillus cereus*	83	60	β
***B*31**	*Bacillus cereus*	75	49	β
***B*32**	*Bacillus thuringiensis*	90	45	β
***B*33**	*Bacillus cereus*	76	60	β
***B*34**	*Bacillus cereus*	86	64	β
***B*35**	*Bacillus sp.*	76	73	γ
***B*36**	*Bacillus cereus*	85	70	β
N37	N/D	80	76	β
***B*38**	*Bacillus cereus*	81	69	β

N39	N/D	86	71	β
N40	N/D	95	69	β
***B*41**	*Bacillus cereus*	89	77	β
***Ps*42**	*Pseudomonas fluorescens*	74	49	β

[1]Letters preceding the isolate numbers indicate the genus of that particular isolate. *B* refers to *Bacillus*, N is not determined, *Ps* is *Pseudomonas*, *Pa* is *Paenibacillus* and U is uncultured bacterium.

[2]N/D stands for not determined.

Table 2 Plant growth promotion and antagonistic traits of the 14 isolates selected as partial- or non-hemolytic.

Name[1]	Isolates	Phosphate	Auxin	Chitinase	Siderophore	Protease	Glucanase
***B*2**	*Bacillus megaterium*	-	-	-	-	-	+
***Ps*3**	*Pseudomonas putida*	-	-	-	+	-	+
***B*4**	*Bacillus flexus*	+	-	-	+	+	+
***B*5**	*Bacillus megaterium*	+	-	-	+	+	+
***B*7**	*Bacillus megaterium*	-	-	-	+	+	+
***Pa*8**	*Paenibacillus polymyxa*	+	+	-	-	-	+
***B*9**	*Bacillus cereus*	-	-	-	-	-	+
***B*12**	*Bacillus subtilis*	+	-	-	+	+	-
***B*13**	*Bacillus subtilis*	+	-	+	+	-	+
***B*22**	*Bacillus megaterium*	-	-	-	+	+	+
***B*23**	*Bacillus megaterium*	+	-	+	-	-	-
***B*24**	*Bacillus cereus*	-	-	+	+	+	+
***B*25**	*Bacillus cereus*	-	-	+	+	+	+
***B*35**	*Bacillus* sp.	-	-	-	-	-	+

[1]Letters preceding the numbers of the isolate indicate the genus of that particular isolate. *B* refers to *Bacillus*, *Ps* to *Pseudomonas* and *Pa* to *Paenibacillus*. + indicates a positive result, - indicates a negative result for each specific assay.

Table S1. Isolates showing ≥60% *Fv* growth inhibition obtained from the large-scale liquid antagonism assay, and the corresponding name of 42 isolates selected for their antagonistic activity in solid medium (see Table 1).

No.	GenBank Accesion	Putative species	Inhibition (%)	Name
1	[3]N/A	*Bacillus megaterium*	87	[1]*B*1
2		[2]N/D	67	
3	N/A	*Bacillus megaterium*	88	*B*2
4	JQ830008	*Bacillus megaterium*	95	
5	JQ830014	*Bacillus bataviensis*	75	
6	JQ830019	*Bacillus megaterium*	77	
7	JQ830029	*Pseudomonas putida*	93	
8	JQ830047	*Enterobacter cloacae*	70	
9	JQ830055	*Enterobacter cloacae*	73	
10	JQ830057	*Enterobacter cloacae*	72	
11	JQ829806	*Bacillus megaterium*	91	
12		N/D	63	
13	JQ829835	*Bacillus subtilis*	65	
14	JQ829857	*Bacillus megaterium*	76	
15	N/A	*Bacillus cereus*	86	
16	N/A	*Bacillus megaterium*	67	
17	N/A	*Bacillus megaterium*	86	
18	JQ829897	*Bacillus flexus*	91	
19	JQ829904	*Bacillus pumilus*	72	
20	JQ829909	*Pseudomonas putida*	84	*Ps*3
21	JQ829910	*Bacillus subtilis*	90	
22	JQ829915	*Bacillus flexus*	77	
23	JQ829937	*Paenibacillus lautus*	83	
24	JQ829951	*Bacillus megaterium*	78	
25	JQ829965	*Bacillus flexus*	76	
26	JQ830750	*Bacillus subtilis*	87	
27	JQ830820	*Bacillus megaterium*	87	
28	JQ830822	*Bacillus flexus*	89	*B*4
29	JQ830824	*Bacillus flexus*	68	
30	JQ830832	*Bacillus megaterium*	91	*B*5
31	JQ830833	*Bacillus pumilus*	84	
32	JQ830840	*Bacillus megaterium*	72	
33	JQ830841	*Bacillus megaterium*	79	
34	JQ830856	*Bacillus subtilis*	69	
35	JQ830865	*Enterobacter cloacae*	76	
36	JQ830887	*Bacillus megaterium*	68	
37	JQ830889	*Bacillus megaterium*	68	

38	JQ830897	*Bacillus megaterium*	66	*B*6
39	JQ830907	*Bacillus niacini*	79	
40	JQ830909	*Bacillus cereus*	79	
41	JQ830911	*Bacillus megaterium*	75	
42	JQ830916	*Bacillus megaterium*	71	
43	JQ830925	*Bacillus megaterium*	85	
44	JQ830928	*Bacillus flexus*	81	
45	JQ830931	*Bacillus cereus*	71	
46	JQ830944	*Bacillus subtilis*	87	
47	JQ830955	*Bacillus anthracis*	88	
48	JQ830963	*Bacillus thuringiensis*	69	
49	JQ830964	*Bacillus megaterium*	69	
50	JQ830967	*Bacillus cereus*	88	
51	JQ830974	*Bacillus flexus*	85	
52	JQ831553	*Bacillus cereus*	72	
53	JQ831562	*Bacillus endophyticus*	66	
54	JQ831570	*Bacillus megaterium*	72	
55	JQ831576	*Bacillus cereus*	79	
56	JQ831587	*Bacillus thuringiensis*	72	
57	JQ831600	*Bacillus subtilis*	73	
58	JQ831601	*Bacillus megaterium*	72	
59	JQ831612	*Bacillus megaterium*	75	
60	JQ831623	*Bacillus subtilis*	74	
61	JQ831635	*Bacillus cereus*	79	
62	JQ831638	*Bacillus pumilus*	70	
63	JQ831642	*Bacillus subtilis*	93	
64	JQ831736	*Bacillus licheniformis*	71	
65	JQ831744	*Enterobacter hormaechei*	72	
66	JQ831769	*Bacillus megaterium*	81	
67	JQ831775	*Enterobacter sp.*	77	
68	JQ831778	*Bacillus megaterium*	74	*B*7
69	JQ831799	*Bacillus megaterium*	86	
70	JQ832197	*Bacillus flexus*	92	
71		N/D	82	
72	JQ832235	*Bacillus megaterium*	82	
73	N/A	*Bacillus cereus*	72	
74	N/A	*Bacillus subtilis*	75	
75	JQ832273	*Bacillus axarquiensis*	72	
76	N/A	Sequence not found	75	
77	JQ832289	*Bacillus oceanisediminis*	71	
78	JQ832292	*Bacillus flexus*	92	
79	JQ832294	*Bacillus megaterium*	83	
80	JQ832300	*Bacillus endophyticus*	77	
81	JQ832303	*Bacillus megaterium*	72	
82		N/D	71	

83	JQ832318	*Bacillus megaterium*	73	
84	JQ832359	*Bacillus megaterium*	85	
85	JQ832391	*Bacillus flexus*	61	
86	JQ832407	*Bacillus megaterium*	63	
87	N/A	*Paenibacillus polymyxa*	62	*Pa*8
88	JQ832940	*Bacillus subtilis*	55	
89	JQ832950	*Bacillus endophyticus*	62	
90	JQ832951	*Bacillus firmus*	53	
91	JQ832967	*Bacillus megaterium*	65	
92	N/A	*Bacillus cereus*	62	*B*9
93	JQ832994	*Bacillus flexus*	90	
94	JQ833012	*Bacillus subtilis*	92	
95	JQ833042	*Acinetobacter lwoffii*	92	
96	JQ833045	*Bacillus megaterium*	90	
97	JQ833055	*Bacillus flexus*	79	
98	JQ833056	*Klebsiella pneumoniae*	82	
99	JQ833058	*Bacillus flexus*	83	
100	JQ833076	*Bacillus subtilis*	73	
101		N/D	85	
102	JQ833115	*Bacillus megaterium*	85	
103	JQ833378	*Bacillus subtilis*	81	
104	JQ833407	*Bacillus pumilus*	73	
105	JQ833429	*Pseudomonas lini*	90	
106	JQ833437	*Pseudomonas corrugata*	71	
107	JQ833466	*Pseudomonas lini*	90	
108	JQ833494	*Pseudomonas lini*	88	
109	JQ833497	*Pseudomonas corrugata*	82	
110	JQ833513	*Pseudomonas corrugata*	76	
111	JQ833514	*Pseudomonas corrugata*	70	
112	JQ833520	*Bacillus cereus*	90	
113	JQ833534	*Pseudomonas lini*	89	
114	JQ833544	*Pseudomonas corrugata*	82	
115	JQ833545	*Bacillus cereus*	83	*B*10
116	JQ833582	*Pseudomonas corrugata*	90	
117	JQ833593	*Bacillus thuringiensis*	70	
118	JQ833598	*Pseudomonas corrugata*	90	
119	JQ833613	*Pseudomonas lini*	90	
120	JQ833616	*Bacillus pumilus*	93	
121	JQ834168	*Bacillus megaterium*	98	
122		N/D	84	N11
123	JQ834184	*Bacillus cereus*	80	
124	JQ834187	*Bacillus subtilis*	95	
125	JQ834196	*Bacillus megaterium*	91	
126	JQ834198	*Bacillus subtilis*	79	*B*12
127	JQ834212	*Bacillus anthracis*	91	

128	JQ834214	*Bacillus megaterium*	81	
129	JQ834233	*Brevibacillus sp.*	71	
130	JQ834013	*Bacillus subtilis*	94	
131	JQ834018	*Bacillus subtilis*	86	*B*13
132	JQ834071	*Bacillus megaterium*	94	
133	JQ834077	*Bacillus anthracis*	73	
134	N/A	*Bacillus thuringiensis*	63	
135	JQ834270	*Bacillus subtilis*	89	
136	JQ834282	*Bacillus subtilis*	71	
137	JQ834304	*Bacillus thuringiensis*	81	*B*14
138		N/D	96	
139	JQ834357	*Bacillus megaterium*	95	
140	JQ834367	*Bacillus megaterium*	66	
141	JQ834358	*Bacillus subtilis*	66	
142	N/A	*Bacillus cereus*	67	
143	JQ834391	*Bacillus megaterium*	68	
144	JQ834392	*Bacillus subtilis*	70	
145	JQ834376	*Bacillus megaterium*	85	
146	JQ834406	*Bacillus marisflavi*	87	
147	JQ834353	*Bacillus cereus*	74	
148		N/D	94	
149	JQ834461	*Bacillus subtilis*	73	
150	JQ834464	*Bacillus cereus*	93	
151	JQ834465	*Bacillus anthracis*	89	
152	JQ834482	*Bacillus cereus*	67	
153	JQ834483	*Bacillus subtilis*	73	
154	JQ834502	*Bacillus cereus*	67	
155	JQ834508	*Lysinibacillus sp*	68	
156	JQ834953	*Bacillus thuringiensis*	67	
157	JQ834955	*Lysinibacillus sphaericus*	85	
158	JQ834960	*Bacillus megaterium*	79	
159	JQ834970	*Bacillus pumilus*	85	
160	JQ834977	*Bacillus badius*	80	
161	JQ834995	*Bacillus endophyticus*	70	
162		N/D	73	
163	N/A	*Bacillus thuringiensis*	71	
164	JQ835012	*Bacillus subtilis*	74	
165	JQ835021	*Lysinibacillus sp.*	69	
166	JQ835027	*Bacillus flexus*	83	
167	JQ835031	*Bacillus cereus*	80	*B*15
168	JQ835033	*Bacillus flexus*	80	
169	JQ835034	*Bacillus flexus*	86	
170		N/D	67	
171	N/A	*Lysinibacillus sp.*	77	
172	JQ835053	*Bacillus megaterium*	82	

173	JQ835057	*Lysinibacillus sphaericus*	71	
174	JQ835064	*Bacillus marisflavi*	85	
175	N/A	*Bacillus fusiformis*	98	
176	JQ835086	*Lysinibacillus fusiformis*	74	
177	JQ835101	*Lysinibacillus fusiformis*	75	
178	JQ835102	*Bacillus megaterium*	69	
179	N/A	*Bacillus oceanisediminis*	89	
180	JQ835139	*Bacillus endophyticus*	68	
181		N/D	72	
182	JQ835141	*Bacillus megaterium*	95	
183	JQ835683	*Bacillus megaterium*	74	
184	JQ835677	*Bacillus flexus*	91	
185	JQ835690	*Bacillus flexus*	80	
186	JQ835695	*Bacillus flexus*	80	
187	JQ835705	*Bacillus thuringiensis*	82	
188		N/D	73	
189	JQ835788	*Bacillus thuringiensis*	71	
190		N/D	91	
191	JQ835763	*Bacillus flexus*	69	
192	JQ835802	*Bacillus megaterium*	63	
193		N/D	66	
194		N/D	66	
195		N/D	82	
196		N/D	96	
197		N/D	90	
198	N/A	*Bacillus subtilis*	81	*B*16
199		N/D	84	
200		N/D	80	
201		N/D	81	
202		N/D	79	
203		N/D	92	
204		N/D	90	
205		N/D	84	
206		N/D	73	
207		N/D	89	
208		N/D	71	
209	JQ829171	*Bacillus flexus*	83	
210	JQ829195	*Bacillus megaterium*	79	
211	JQ829196	*Bacillus flexus*	68	
212	JQ829207	*Bacillus aryabhattai*	77	
213	N/A	*Aquaspirillum itersonii*	66	
214	JQ830117	*Bacillus flexus*	69	
215	JQ830122	*Bacillus flexus*	96	
216	JQ830123	*Bacillus megaterium*	70	
217	N/A	*Terribacillus sp.*	70	

218	JQ830179	*Terribacillus sp.*	71	
219	JQ830196	*Bacillus pumilus*	80	
220	JQ830209	*Bacillus flexus*	71	
221	JQ830210	*Bacillus megaterium*	78	
222	JQ830214	*Bacillus megaterium*	74	
223	JQ830215	*Bacillus megaterium*	90	
224	JQ830223	*Bacillus megaterium*	88	
225	JQ830226	*Paenibacillus borealis*	80	
226	JQ830227	*Bacillus niacini*	86	
227	N/A	*Acinetobacter calcoaceticus*	70	
228	JQ830236	*Bacillus megaterium*	72	
229	JQ830237	*Bacillus megaterium*	76	
230	N/A	*Bacillus subtilis*	82	*B*17
231	JQ830258	*Bacillus megaterium*	79	
232	JQ830259	*Bacillus megaterium*	76	
233	JQ830261	*Bacillus subtilis*	81	
234	JQ830268	*Bacillus megaterium*	81	
235	JQ830269	*Bacillus megaterium*	77	
236	JQ830273	*Bacillus megaterium*	70	
237	JQ830277	*Lysinibacillus fusiformis*	82	
238	JQ830278	*Bacillus megaterium*	88	
239	JQ830279	*Bacillus megaterium*	91	
240	JQ830281	*Bacillus megaterium*	50	
241	JQ830287	*Bacillus megaterium*	72	
242	JQ830299	*Bacillus cereus*	89	
243	JQ830308	*Bacillus licheniformis*	92	
244		N/D	81	
245		N/D	80	
246		N/D	97	
247		N/D	82	
248		N/D	74	
249		N/D	83	
250		N/D	88	
251		N/D	87	
252		N/D	91	
253		N/D	87	
254	N/A	*Bacillus thurigiensis*	85	*B*18
255	N/A	*Bacillus thiringiensis*	71	*B*19
256	JQ830993	*Bacillus megaterium*	86	
257	JQ830999	*Bacillus thuringiensis*	97	
258	JQ831023	*Bacillus flexus*	99	
259	JQ831032	*Bacillus pumilus*	90	
260	JQ831035	*Bacillus endophyticus*	85	
261	JQ831037	*Bacillus badius*	84	
262	JQ831050	*Bacillus badius*	84	

263	JQ831055	*Bacillus megaterium*	81	
264	JQ831060	*Bacillus thuringiensis*	85	*B20*
265	JQ831062	*Bacillus megaterium*	86	
266	JQ831069	*Bacillus thuringiensis*	91	
267	JQ831071	*Bacillus megaterium*	86	
268	JQ831073	*Bacillus megaterium*	82	
269		N/D	85	
270		N/D	83	
271		N/D	94	
272		N/D	92	
273		N/D	89	
274		N/D	74	
275		N/D	73	
276		N/D	70	
277		N/D	78	
278		N/D	79	
279		N/D	83	
280	N/A	*Bacillus thuringiensis*	82	*B*21
281	N/A	*Bacillus megaterium*	74	*B*22
282		N/D	73	
283		N/D	80	
284		N/D	71	
285		N/D	83	
286		N/D	76	
287		N/D	75	
288		N/D	71	
289		N/D	90	
290		N/D	74	
291		N/D	71	
292		N/D	71	
293		N/D	91	
294		N/D	79	
295		N/D	94	
296		N/D	84	
297		N/D	71	
298		N/D	71	
299		N/D	81	
300		N/D	81	
301		N/D	72	
302		N/D	88	
303	JQ832079	*Bacillus flexus*	80	
304	JQ832081	*Bacillus megaterium*	76	
305	JQ832083	*Bacillus cereus*	83	
306	JQ832089	*Bacillus megaterium*	86	
307	JQ832091	*Bacillus thuringiensis*	85	

308	JQ832092	*Bacillus cereus*	88	
309	JQ832097	*Bacillus megaterium*	79	
310	JQ832106	*Bacillus megaterium*	78	
311	JQ832111	*Bacillus megaterium*	77	
312	JQ832118	*Bacillus megaterium*	83	
313		N/D	79	
314	JQ832123	*Bacillus megaterium*	82	
315	JQ832124	*Bacillus megaterium*	83	
316		N/D	80	
317	JQ832141	*Bacillus flexus*	78	
318	JQ832142	*Bacillus flexus*	76	
319	JQ832143	*Bacillus megaterium*	79	
320	JQ832820	*Bacillus megaterium*	76	
321	JQ832829	*Bacillus cereus*	63	
322	JQ832423	*Bacillus flexus*	70	
323	JQ832418	*Bacillus megaterium*	64	*B*23
324	JQ832488	*Bacillus subtilis*	94	
325	JQ832499	*Enterobacter cloacae*	65	
326	JQ832567	*Lysinibacillus fusiformis*	62	
327	JQ832569	*Enterobacter cloacae*	64	
328		N/D	77	
329		N/D	62	
330	JQ833149	*Bacillus subtilis*	90	
331	JQ833162	*Bacillus megaterium*	87	
332	JQ833144	*Bacillus megaterium*	81	
333	JQ833201	*Lysinibacillus fusiformis*	73	
334	JQ833287	*Bacillus cereus*	62	
335	JQ833280	*Bacillus megaterium*	60	
336	JQ833303	*Bacillus megaterium*	65	
337	JQ833707	*Bacillus flexus*	68	
338	JQ833672	*Bacillus megaterium*	70	
339	JQ833675	*Bacillus megaterium*	67	
340	JQ833765	*Bacillus megaterium*	61	
341	JQ833789	*Bacillus flexus*	75	
342	JQ833756	*Bacillus cereus*	67	
343	N/A	*Anaerobranca californiensis*	87	
344	JQ833917	*Bacillus megaterium*	82	
345	JQ834598	*Bacillus subtilis*	72	
346	JQ834607	*Bacillus megaterium*	89	
347	JQ834649	*Pseudomonas stutzeri*	85	
348	JQ834672	*Bacillus cereus*	86	
349	JQ834691	*Bacillus megaterium*	79	
350	JQ834737	*Bacillus megaterium*	77	
351	JQ835192	*Bacillus cereus*	70	
352	JQ835234	*Bacillus subtilis*	73	

353	JQ835299	*Bacillus pumilus*	74	
354	JQ835289	*Bacillus megaterium*	78	
355	JQ835291	*Bacillus megaterium*	76	
356	JQ835314	*Bacillus aryabhattai*	74	
357	JQ835336	*Bacillus megaterium*	73	
358	JQ835385	*Bacillus megaterium*	82	
359	JQ835396	*Bacillus megaterium*	74	
360	JQ835403	*Lysinibacillus fusiformis*	81	
361	JQ835408	*Bacillus cereus*	75	
362	JQ835418	*Bacillus megaterium*	89	
363	JQ835429	*Bacillus megaterium*	80	
364	JQ835429	*Bacillus thuringiensis*	72	
365	JQ835834	*Bacillus thuringiensis*	72	
366	JQ835838	*Bacillus thuringiensis*	73	
367	JQ835859	*Bacillus cereus*	72	*B*24
368	JQ835870	*Bacillus cereus*	72	
369	JQ835881	*Lysinibacillus fusiformis*	72	
370	JQ835905	*Lysinibacillus fusiformis*	76	
371	JQ835875	*Pseudomonas chlororaphis*	76	
372	JQ835919	*Bacillus flexus*	92	
373	JQ835946	*Bacillus cereus*	93	*B*25
374	JQ835949	*Bacillus thuringiensis*	86	
375	JQ835950	*Bacillus thuringiensis*	89	
376	JQ835987	*Bacillus cereus*	92	
377	JQ835998	*Bacillus cereus*	91	
378		N/D	88	
379		N/D	87	
380		N/D	90	
381	JQ829267	*Enterobacter asburiae*	90	
382	JQ829276	*Bacillus pumilus*	88	
383	JQ829288	*Enterobacter hormaechei*	91	
384	JQ829294	*Enterobacter cloacae*	89	
385	JQ829301	*Enterobacter hormaechei*	91	
386	JQ829310	*Enterobacter cloacae*	90	
387	JQ829353	*Enterobacter cloacae*	93	
388	JQ829362	*Enterobacter hormaechei*	92	
389	JQ829425	*Enterobacter cloacae*	75	
390	JQ829426	*Enterobacter asburiae*	93	
391	JQ829479	*Enterobacter asburiae*	83	
392	JQ829460	*Enterobacter hormaechei*	84	
393	JQ829461	*Enterobacter hormaechei*	87	
394	JQ829518	*Enterobacter hormaechei*	82	
395	JQ830336	*Bacillus thuringiensis*	82	
396	JQ830343	*Enterobacter hormaechei*	84	
397	JQ830356	*Enterobacter cancerogenus*	82	

398	JQ830403	*Bacillus arbutinivorans*	85	
399	JQ830426	*Enterobacter hormaechei*	79	
400	JQ830443	*Enterobacter asburiae*	86	
401	JQ830486	*Enterobacter asburiae*	87	
402	JQ830498	*Enterobacter aerogenes*	81	
403	JQ830534	*Enterobacter hormaechei*	75	
404	JQ831079	*Stenotrophomonas maltophilia*	71	
405	JQ831086	*Bacillus oceanisediminis*	72	
406	JQ831089	*Bacillus pumilus*	75	
407		N/D	83	
408	JQ831141	*Arthrobacter globiformis*	74	
409	JQ831159	*Agrobacterium tumefaciens*	75	
410	JQ831175	*Bacillus pumilus*	77	
411	JQ831176	*Bacillus pumilus*	87	
412	JQ831182	*Pseudomonas pseudoalcaligenes*	78	
413	N/A	*Bacillus pumilus*	85	
414	N/A	Uncultured bacterium clone	87	
415	JQ831198	*Bacillus altitudinis*	90	
416	JQ831199	*Agrobacterium rubi*	90	
417	JQ831210	*Bacillus pumilus*	79	
418	JQ831216	*Bacillus cereus*	82	
419	N/A	*Brevibacillus brevis*	79	
420	N/A	*Bacillus drentensis*	81	
421	JQ831242	*Stenotrophomonas maltophilia*	83	
422	JQ831248	*Bacillus cereus*	74	*B*26
423	JQ831249	*Bacillus cereus*	77	
424	N/A	*Stenotrophomonas maltophilia*	92	
425		N/D	75	N27
426	JQ831276	*Paenibacillus xylanilyticus*	82	
427	JQ831280	*Bacillus megaterium*	92	
428	JQ831284	*Stenotrophomonas maltophilia*	79	
429	JQ831294	*Agrobacterium tumefaciens*	88	
430	N/A	*Bacillus* sp.	76	*B*35
431		N/D	77	
432		N/D	74	
433		N/D	75	
434		N/D	73	
435		N/D	92	
436	N/A	*Bacillus pumilus*	82	
437	JQ831833	*Bacillus cereus*	94	
438		N/D	86	
439	JQ831889	*Bacillus megaterium*	76	
440		N/D	84	
441	JQ831907	*Arthrobacter globiformis*	92	

442	N/A	*Bacillus thuringiensis*	75	
443	JQ831917	*Bacillus flexus*	82	
444	N/A	*Bacillus cereus*	89	
445		N/D	94	
446	JQ832587	*Bacillus cereus*	79	*B*28
447		N/D	89	
448	JQ832589	*Bacillus thuringiensis*	77	
449		N/D	76	
450	JQ832600	*Bacillus oceanisediminis*	83	
451	JQ832601	*Bacillus cereus*	72	*B*29
452		N/D	77	
453		N/D	80	
454	JQ832624	*Bacillus oceanisediminis*	76	
455	N/A	*Bacillus cereus*	89	
456	JQ832645	*Bacillus cereus*	92	
457	JQ832668	*Bacillus cereus*	83	*B*30
458	JQ832678	*Bacillus cereus*	75	
459	JQ832688	*Bacillus cereus*	79	
460	JQ832702	*Bacillus cereus*	75	*B*31
461		N/D	80	
462	N/A	*Bacillus thuringiensis*	90	*B*32
463		N/D	81	
464		N/D	79	
465		N/D	80	
466		N/D	85	
467		N/D	80	
468		N/D	85	
469		N/D	85	
470		N/D	85	
471		N/D	87	
472		N/D	86	
473		N/D	87	
474	N/A	*Bacillus cereus*	86	*B*34
475		N/D	93	
476		N/D	79	
477		N/D	90	
478		N/D	84	
479	JQ833348	*Arthrobacter globiformis*	85	
480	JQ833929	*Bacillus cereus*	76	*B*33
481	N/A	*Bacillus pumilus*	86	
482	JQ833955	*Bacillus cereus*	78	
483	JQ833957	*Bacillus thuringiensis*	74	
484	N/A	*Geobacillus thermodenitrificans*	78	
485	N/A	*Bacillus cereus*	78	
486		N/D	82	

487	JQ833976	*Bacillus bataviensis*	77	
488	JQ833988	*Bacillus thuringiensis*	77	
489		*N/D*	79	
490		*N/D*	81	
491		*N/D*	79	
492		*N/D*	78	
493	N/A	*Bacillus cereus*	85	*B*36
494		*N/D*	75	
495		*N/D*	80	N37
496		*N/D*	78	
497		*N/D*	76	
498		*N/D*	78	
499	N/A	*Bacillus cereus*	81	*B*38
500		N/D	77	
501		N/D	75	
502		N/D	75	
503		N/D	84	
504		N/D	76	
505		N/D	83	
506		N/D	87	
507		N/D	91	
508		N/D	84	
509	JQ834802	*Bacillus cereus*	86	
510	JQ834819	*Bacillus cereus*	81	
511	JQ834825	*Bacillus cereus*	77	
512	JQ834832	*Bacillus cereus*	81	
513	JQ834842	*Bacillus cereus*	95	
514	N/A	Uncultured bacterium clone	83	
515	N/A	*Bacillus cereus*	79	
516		N/D	78	
517	JQ834824	*Bacillus firmus*	76	
518		N/D	85	
519		N/D	77	
520	N/A	*Bacillus cereus*	91	
521	JQ835485	*Bacillus cereus*	79	
522	JQ835547	*Bacillus pumilus*	78	
523	JQ835589	*Bacillus cereus*	76	
524	JQ835602	*Bacillus cereus*	84	
525	JQ835641	*Pseudomonas putida*	77	
526		N/D	86	N39
527		N/D	96	
528	N/A	*Bacillus asahii*	77	
529	N/A	*Acinetobacter rhizosphaerae*	81	
530	JQ836137	*Acinetobacter calcoaceticus*	89	
531		N/D	88	

532	N/A	*Bacillus cereus*	90	
533	JQ836158	*Bacillus thuringiensis*	80	
534	N/A	*Bacillus cereus*	84	
535	JQ836159	*Bacillus sonorensis*	85	
536	N/A	*Bacillus cereus*	85	
537	N/A	*Bacillus thuringiensis*	94	
538		N/D	87	
539	N/A	*Bacillus licheniformis*	91	
540	JQ836172	*Bacillus cereus*	81	
541	JQ836173	*Bacillus cereus*	84	
542		N/D	95	N40
543	N/A	*Bacillus cereus*	89	*B*41
544	JQ836198	*Bacillus cereus*	89	
545	JQ829546	*Pantoea dispersa*	78	
546	JQ829551	*Pantoea dispersa*	83	
547	JQ829555	*Bacillus licheniformis*	89	
548	JQ829577	*Pantoea dispersa*	78	
549	JQ829580	*Bacillus cereus*	98	
550	JQ829606	*Bacillus megaterium*	80	
551	JQ829658	*Bacillus megaterium*	87	
552	JQ829661	*Bacillus flexus*	86	
553	JQ829684	*Bacillus megaterium*	93	
554	JQ829694	*Bacillus megaterium*	87	
555	JQ829752	*Bacillus cereus*	79	
556		N/D	82	
557	JQ829755	*Bacillus megaterium*	76	
558	JQ829723	*Bacillus cereus*	90	
559		N/D	96	
560		N/D	88	
561		N/D	85	
562		N/D	91	
563		N/D	88	
564		N/D	91	
565		N/D	86	
566		N/D	92	
567		N/D	94	
568		N/D	89	
569		N/D	89	
570		N/D	90	
571		N/D	92	
572		N/D	90	
573	JQ830598	*Enterobacter cloacae*	89	
574	JQ830601	*Enterobacter cloacae*	87	
575	JQ830608	*Enterobacter cloacae*	91	
576	JQ830629	*Enterobacter cloacae*	84	

577	N/A	*Bacillus subtilis*	85	
578	JQ830649	*Enterobacter cloacae*	86	
579	JQ830665	*Enterobacter cloacae*	88	
580	JQ830666	*Enterobacter cloacae*	89	
581	JQ830689	*Enterobacter sp.*	89	
582	JQ830696	*Klebsiella pneumonae*	89	
583	JQ830697	*Enterobacter cloacae*	88	
584		N/D	92	
585	JQ830734	*Bacillus subtilis*	88	
586	JQ831337	*Bacillus megaterium*	88	
587	N/A	*Bacillus cereus*	87	
588		N/D	90	
589	JQ831449	*Enterobacter ludwigii*	90	
590		N/D	75	
591		N/D	76	
592		N/D	74	
593		N/D	88	
594		N/D	74	
595		N/D	59	
596		N/D	89	
597		N/D	93	
598		N/D	93	
599	N/A	*Bacillus cereus*	66	
600		N/D	69	
601		N/D	75	
602		N/D	79	
603	JQ831992	*Bacillus cereus*	70	
604	JQ832028	*Bacillus megaterium*	72	
605	JQ832030	*Bacillus subtilis*	79	
606	JQ832046	*Bacillus megaterium*	60	
607		N/D	60	
608		N/D	70	
609		N/D	60	
610		N/D	85	
611		N/D	95	
612		N/D	70	
613		N/D	63	
614		N/D	72	
615		N/D	81	
616		N/D	60	
617	JQ832716	*Pseudomonas fluorescens*	70	
618	JQ832727	*Pseudomonas putida*	60	
619	N/A	*Pseudomonas fluorescens*	74	*Ps*42
620		N/D	60	
621	JQ832755	*Pseudomonas fluorescens*	81	

622	JQ832768	*Pseudomonas putida*	76

[1]Letters preceding the isolate number indicate the genus of that particular isolate. *B* refers to *Bacillus*, N is not determined, *Ps* is *Pseudomonas*, *Pa* is *Paenibacillus* and U is uncultured bacterium.
[2]N/D stands for not determined.
[3]N/A stands for not assigned.

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