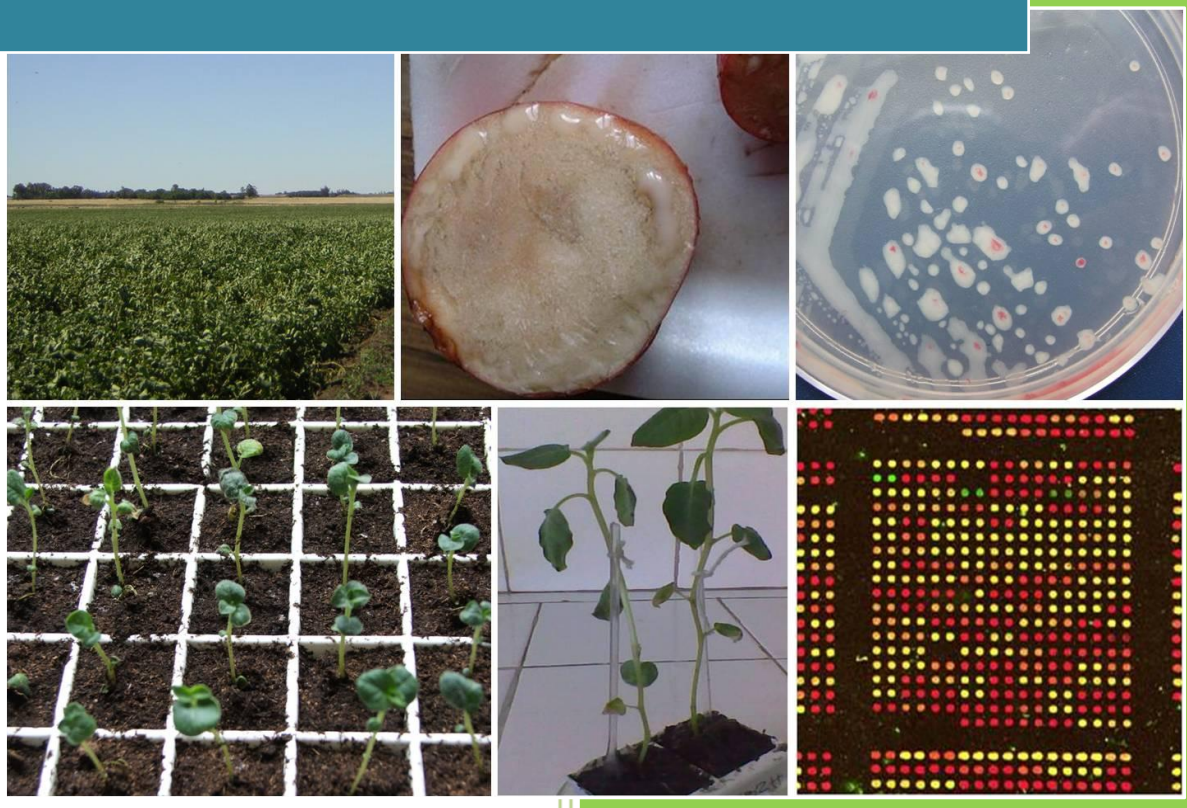
Estudio genómico comparativo de las cepas de Ralstonia solanacearum causantes de la marchitez bacteriana de la papa en Uruguay



María Inés Siri

Estudio genómico comparativo de las cepas de *Ralstonia solanacearum* causantes de la marchitez bacteriana de la papa en Uruguay

María Inés Siri

Trabajo presentado para obtener el título de

DOCTOR EN QUÍMICA

Directores de Tesis :

María Julia Pianzzola

Christian Boucher

Tribunal:

Guillermo Galván

Elena Orellano

Matilde Soubes

Marc Valls

Pablo Zunino

Montevideo

Diciembre de 2010

Universidad de la República

Facultad de Química

PEDECIBA QUÍMICA

AGRADECIMIENTOS

El llevar adelante una tesis de Doctorado sin duda lleva mucho trabajo y dedicación. Al llegar al final de esta etapa y mirar hacia atrás, quiero agradecer a todas las personas que compartieron conmigo este camino haciendo que sea disfrutable y enriquecedor.

En primer lugar, quiero decirle gracias a María Julia, por la confianza y cariño de todos estos años de trabajo juntas. Sus conocimientos, sus orientaciones, su forma de trabajar, su persistencia y su motivación, han sido fundamentales para mi formación como investigadora. Gracias por darme total libertad para explorar mis propios caminos y la contención y ayuda necesarias cada vez que precisé un cable a tierra.

También quiero agradecer a Christian, por abrirme las puertas de su casa sin apenas conocerme, por su amabilidad y disponibilidad durante mis estancias en su grupo, durante las cuales tuve todo el soporte profesional y logístico para alcanzar los objetivos perseguidos. Muchas gracias por permitirme vivir una experiencia tan importante para mi formación.

A todos los organismos que permitieron financiar este trabajo a través de varios proyectos de investigación (PDT) y becas de estudio (PEDECIBA, ANII, CSIC).

A la Embajada de Francia, por otorgarme apoyo económico para cada una de mis estancias en Toulouse, y a Graciela Vigo por toda su amabilidad y ayuda.

A todos mis compañeros y amigos que en estos años he conocido en la Cátedra de Microbiología, por tantos momentos compartidos dentro y fuera de los laboratorios y el ambiente de compañerismo que ha estimulado el trabajo de investigación.

En particular, a mis compañeros del Laboratorio de Microbiología Molecular, es decir del “12”. A Gianna y Sonia, por tantos consejos y charlas enriquecedoras. A la barra joven por hacer del laboratorio un hábitat divertido dónde transcurrir muchas horas del día.

A Analía, gracias por compartir triunfos y desalientos en el intento de saber un poco más sobre nuestra entrañable *Ralstonia.* Gracias por tu sentido natural por el bien común, que en muchas ocasiones me sacó de apuros y fue de gran ayuda para mi trabajo.

A Virginia, mi primera estudiante de grado y recientemente incorporada al equipo *Ralstonia*. Gracias por todo el trabajo dedicado a la evaluación y optimización de los métodos de detección por PCR, que ha sido de gran ayuda en las etapas finales de esta tesis.

A todos los integrantes del Laboratorio de Biotecnología del INIA (Las Brujas), por su ayuda y asesoramiento en el trabajo de propagación y multiplicación *in vitro* de plantas usadas en esta tesis. En especial, mi agradecimiento a Alicia Castillo, por su valioso apoyo en este tema, por su generosidad y consejos de todos estos años.

También a mis compañeros del LIPM-INRA, por las buenas experiencias vividas en cada una de mis instancias en Toulouse, a Stephan, Nemo, Alice, Mounira, Marie, Anne-Claire, Laure, Philippe, Patrick, Ilona, Fabienne: merci! A Lenaick y Adriana por su especial generosidad y amistad incondicionales, gracias por todo el apoyo que me dieron, las conversaciones en español, y los buenos momentos que pasamos juntos.

A Enrique Verdier, por ser mi referente en cuanto a la "historia" de la *Ralstonia* en Uruguay, por proveerme las cepas de 1980, y transmitirme generosamente y con humildad toda su experiencia y conocimientos.

A todo el equipo *commersonii*: Paco, Matías, Marco, Rafa, y en especial a Guille, por estar siempre a mano, por su valiosa ayuda y amistad.

A Lu, mi amiga incondicional, por haber tenido la suerte de recorrer junto a ella todos los caminos.

A mi familia, por el amor y apoyo que siempre me han dado. En especial quiero agradecer a mis padres y a mis suegros, por poder contar con su ayuda como abuelos incondicionales y en sana competencia, sin la cual no habría podido entregarme con toda mi dedicación a esta tesis.

A mis amores, Ignacio y Fede, por ser mi luz, lo más valioso que tengo en la vida.

RESUMEN

Estudio genómico comparativo de las cepas de *Ralstonia solanacearum* causantes de la marchitez bacteriana de la papa en Uruguay

Ralstonia solanacearum es el agente responsable de la *marchitez bacteriana*, una de las enfermedades de plantas más destructivas a nivel mundial. En Uruguay este patógeno constituye uno de los principales problemas sanitarios asociados al cultivo de papa. El objetivo general de esta tesis es generar conocimiento sobre las cepas de *R. solanacearum* que afectan los cultivos de papa en Uruguay, de forma de contribuir al desarrollo de estrategias efectivas para su control. Para ello, se generó una colección de cepas uruguayas de *R. solanacearum* encontrando que todas pertenecen al filotipo IIB, secuevar 1 (IIB-1), un grupo especialmente adaptado a la papa como hospedero y que se asocia a climas templados. Se encontraron diferencias de agresividad entre las cepas sobre varios hospederos incluyendo tomate, papa y la especie silvestre *Solanum commersonii,* una especie silvestre nativa de Uruguay considerada una valiosa fuente de resistencia en programas de mejoramiento de papa. La diversidad genética se evaluó mediante marcadores BOX-PCR y por hibridación genómica comparativa (CGH) usando la tecnología de *microarrays.* Globalmente estos análisis revelaron una baja diversidad genética entre las cepas presentes en Uruguay y confirmaron su clasificación como filotipo IIB, secuevar 1. El análisis genómico comparativo por CGH permitió establecer el contenido genético de cada una de las cepas uruguayas analizadas. Esta información se analizó con dos enfoques diferentes. Por un lado, se comparó el contenido genético entre cepas con diferente nivel de agresividad u origen de aislamiento, identificando genes involucrados en la virulencia de *R. solanacearum.* Por otro lado, los datos generados se utilizaron para establecer una lista de genes específicos de cepas IIB-1, los cuales se aplicaron al desarrollo de métodos de detección específicos que permitan evitar la diseminación del patógeno. Los resultados generados en esta tesis contribuyen a aumentar el valor biológico de los datos de secuencia actualmente disponibles y apuntan a su aprovechamiento para el desarrollo de estrategias de control de este importante patógeno.

ÍNDICE GENERAL

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

CAPÍTULO 1

Ralstonia solanacearum: agente causal de la marchitez bacteriana

INTRODUCCIÓN GENERAL

Ralstonia solanacearum es el agente responsable de la *marchitez bacteriana*, una de las enfermedades de plantas más destructivas a nivel mundial. Este patógeno ha sido reportado en regiones tropicales, sub-tropicales y templadas, y se caracteriza por presentar un rango de hospederos extremadamente amplio que abarca a más de 200 especies distribuidas en más de 50 familias diferentes (Elphinstone 2005; Hayward, 1994b). Otra característica destacada es la gran diversidad que presenta este patógeno, lo que genera una serie de problemas en el manejo de la enfermedad. *R. solanacearum* actualmente es considerado un complejo de especies que reúne un conjunto de cepas que pueden diferenciarse en cuanto a rango de hospederos, distribución geográfica, agresividad, relaciones epidemiológicas, y propiedades fisiológicas (Buddenhagen et al., 1962; Castillo y Greenberg, 2007; Fegan y Prior, 2005; Hayward, 1964; 1994a).

En base a estas características, *R. solanacearum* es reconocido como un microorganismo modelo para el estudio de mecanismos de patogenia e interacción planta-patógeno, así como de aspectos evolutivos y de diversificación de la especie. Dada su importancia a nivel mundial y las dificultades asociadas al control de esta enfermedad, también existe una extensa y activa investigación sobre aspectos más aplicados, incluyendo el mejoramiento genético de los cultivos, métodos de detección y diagnóstico, control biológico y estudios epidemiológicos de dispersión y supervivencia. En este capítulo se presenta una revisión bibliográfica sobre el estado actual del conocimiento en el tema.

Ralstonia solanacearum: taxonomía y clasificación

Las cepas de *R. solanacearum* pertenecen al orden *Burkholderiales* de las β-proteobacterias. Se trata de un bacilo Gram-negativo, aerobio estricto con metabolismo oxidativo y una temperatura óptima de crecimiento entre 28 y 30 °C (Hayward, 1964). El agente responsable de la marchitez bacteriana fue descrito por primera vez por Smith (1896) como *Bacillus solanacearum* y desde entonces ha recibido varias denominaciones. En 1914 fue renombrado por Smith como *Pseudomonas solanacearum*, denominación que mantuvo durante casi 80 años (Skerman et al., 1980). En 1992, durante la reestructuración taxonómica del género *Pseudomonas*, esta bacteria se clasificó como *Burkholderia solanacearum* (Smith) (Yabuuchi et al., 1992) en base a análisis de secuencia del gen 16S ARNr, homología ADN-ADN, y composición de ácidos grasos. Finalmente tres años después, fue nuevamente reclasificada permaneciendo dentro de la misma familia pero en un nuevo género, *Ralstonia*, para albergar un grupo de homología de DNA distinto al de la especie *Burkholderia cepacia* (Yabuuchi et al., 1995). *R. solanacearum* es considerado actualmente como un complejo de especies, que abarca muchos grupos de cepas estrechamente relacionadas pero distintas genéticamente. Dado que estas cepas difieren en el rango de hospederos, distribución geográfica, patogenicidad y fisiología, resulta importante contar con un sistema de clasificación que permita predecir la epidemiología y el uso de métodos apropiados de control (Hayward, 1991).

Históricamente, las cepas de *R. solanacearum* se agruparon en cinco razas según el rango de hospederos susceptibles (Buddenhagen et al., 1962; He et al., 1983; Pegg y Moffett, 1971) y en seis biovares en base a propiedades bioquímicas (Hayward, 1964, 1994a). La raza 1 constituye el grupo más heterogéneo en lo que refiere a su rango de hospederos, siendo capaz de afectar a una amplia variedad de plantas, incluyendo tomate, papa, berenjena, tabaco, oliva, maní, jengibre, y algunas especies no cultivadas. La raza 2 está asociada específicamente a la familia de las bananas (*Musaceae*) y a algunas especies del género *Heliconia*. La raza 3 afecta principalmente papa y tomate pero actualmente se determinó que tiene un rango más amplio de hospederos, incluyendo berenjena, geranio, repollo, y varias malezas. Las razas 4 y 5 están asociadas a jengibre y morera respectivamente. A su vez, dentro de cada raza

pueden existir cepas con diferente nivel de agresividad para cultivares de una misma especie hospedera (Jaunet y Wang, 1999; Mc.Laughlin y Sequeira, 1989; Prior et al., 1990). A lo largo de los años, la diversidad de este complejo de especies ha ido aumentando y este sistema de clasificación basado en el rango de hospederos ha demostrado ser ambiguo y poco fiable para predecir el potencial patogénico de *R. solanacearum* (Denny et al., 2006).

El sistema de biovar originalmente propuesto por Hayward (1964) agrupa las cepas según su capacidad de producir ácido a partir de la oxidación de tres disacáridos (celobiosa, lactosa y maltosa) y tres alcoholes hexosa (dulcitol, manitol y sorbitol). El sistema se amplió posteriormente para incluir otros sustratos, determinando la existencia de 6 biovares (Hayward, 1994a). Inicialmente fueron reconocidos cuatro biovares (1- 4) abarcando a la mayoría de las cepas aisladas hasta el momento. Un estudio más detallado sobre una colección de aislamientos de *R. solanacearum* reveló importantes diferencias de algunos aislamientos pertenecientes al biovar 2 respecto al fenotipo original lo que llevó a introducir una nueva diferenciación (Hayward, 1994a). Este nuevo grupo de cepas metabólicamente más activas y con un rango de hospederos más amplio se conoce como 2-T, mientras que el biovar 2 original también se puede referir como 2-A. Finalmente, el biovar 5 fue creado para contemplar a las cepas aisladas de morera en China (Hayward, 1994a).

No existe una correlación general entre biovares y razas, salvo para las cepas de raza 3 que se corresponden con el biovar 2 (2-A) y las de raza 5 que son por lo general del biovar 5. Además, este sistema de clasificación, tampoco tiene la capacidad de ser predictivo sobre la biología y potencial patogénico de las cepas, ya que por ejemplo, las cepas de biovares 1 y 3 pueden ser aisladas de hospederos muy variados. Sin embargo, este método ha sido ampliamente adoptado como un rasgo esencial en la caracterización de las cepas de *R. solanacearum* dado que resulta fácil, barato y reproducible, por lo que se continúa usando en muchos laboratorios (Denny, 2006).

En las últimas décadas se han logrado importantes avances en la clasificación y caracterización de *R. solanacearum* utilizando métodos moleculares, lo que ha contribuido al conocimiento sobre los aspectos evolutivos de este patógeno. El uso de RFLP (*Restriction Fragment Length Polymorphism*) permitió dividir la especie en dos

grupos que se correlacionan con el origen de dispersión geográfica de las cepas: la división "Asiática" (división I) comprende cepas de los biovares 3, 4 y 5 mientras que la división "Americana" (división II) contiene cepas de los biovares 1, 2-A y 2-T (Cook et al., 1989). Posteriormente, se describió un tercer grupo al incorporar en el estudio cepas originarias de África (Poussier et al., 1999).

En 2005 se propuso un nuevo esquema de clasificación para *R. solanacearum* que permitió evidenciar cómo se relacionan las distintas cepas dentro de complejo de especies, generando un sistema coherente y predictivo que es el que se adopta actualmente (Fegan y Prior, 2005). Este sistema permitió identificar cuatro grupos principales denominados "filotipos" en base al análisis de la secuencia del espaciador interno transcrito (ITS) localizado entre los genes 16S - 23S del ARNr. En base a estas secuencias, se diseñó una reacción de multiplex-PCR a través de la cual se puede asignar rápidamente el filotipo a una cepa dada. Los filotipos están relacionados con el origen filogenético y geográfico de las cepas: el filotipo I agrupa los aislamientos de Asia y Australia y comprende a los biovares 3, 4 y 5, el filotipo II incluye cepas de América pertenecientes a los biovares 1, 2 y 2-T, el filotipo III corresponde a cepas de África de los biovares 1 y 2-T, y el filotipo IV está representado por cepas de Indonesia de biovar 1, 2 y 2-T. En este filotipo también se incluyen las especies relacionadas *R. syzigii* causante de la enfermedad de Sumatra en clavo de olor y el patógeno de banana *R. celebensis,* también conocido como BDB (*Blood Disease Bacterium*). A su vez, cada filotipo está conformado por varios "secuevares", definidos como grupos de cepas que presentan una secuencia altamente conservada dentro de una región determinada del genoma. Para el caso de *R. solanacearum*, los secuevares generalmente se definen en base a secuencias parciales del gen de la endoglucanasa (*egl*). Cada secuevar puede a su vez comprender diferentes "líneas clonales" identificadas mediante técnicas de marcadores moleculares como AFLP (*Amplified Fragment Length Polymorphism*), PFGE (*Pulsed Field Gel Electrophoresis*) o rep-PCR.

Dada la posibilidad de analizar simultáneamente cuatro niveles taxonómicos diferentes, esta nueva forma de clasificación de la especie permite añadir nuevos genotipos a medida que son descubiertos, ofreciendo una herramienta muy flexible e informativa para el estudio de la enorme complejidad que caracteriza a este patógeno.

La información así generada y organizada puede ser de gran utilidad para la predicción de las propiedades biológicas de nuevas cepas aisladas de diversos ambientes y hospederos (Fegan y Prior, 2005).

Esta distribución en cuatro grupos principales ha sido confirmada mediante otros enfoques metodológicos lo que demuestra la solidez y la relevancia biológica de este sistema de clasificación (Guidot et al., 2007; Sánchez Pérez et al. 2008; Villa et al. 2005). Esto sugiere que los filotipos reflejan verdaderos linajes evolutivos que probablemente surgieron a través del aislamiento geográfico de los respectivos progenitores y su posterior adaptación a diferentes ambientes y hospederos (Denny, 2006).

En los últimos años han surgido nuevas propuestas en cuanto a la organización taxonómica de este heterogéneo complejo de especies. Según los últimos avances obtenidos por secuenciación del genoma completo de varias cepas de *R. solanacearum* y estudios de hibridación genómica comparativa sobre *microarrays*, se encontró suficiente variación como para que el complejo sea reclasificado en al menos tres grupos taxonómicos, con al menos un 30% de divergencia a nivel de hibridación ADN-ADN. En este nuevo escenario el complejo podría dividirse en tres especies: una conteniendo a las cepas del filotipo II, otra que agruparía a los filotipos I y III, y una tercer especie formada por las cepas de *R. solanacearum* pertenecientes al filotipo IV (Remenant et al., 2010).

El ciclo de vida de *R. solanacearum*

R. solanacearum es un patógeno de suelo, y como tal debe ser capaz de resistir factores de estrés ambiental, asociados al pH, temperatura, disponibilidad de agua y limitación de oxígeno, además de la competencia por los nutrientes. En el corto plazo, la supervivencia en el suelo está controlada principalmente por la humedad y la temperatura. Otros factores que también pueden influir son el tipo de suelo, el contenido de materia orgánica, el nivel de nutrientes, y la biota microbiana presente (Coutinho, 2005, Hayward, 1991). Sin embargo, aún no se conoce exactamente que tipo de suelos o condiciones ambientales son más favorables para la supervivencia de *R. solanacearum* y el posterior desarrollo de la enfermedad en los cultivos. La

supervivencia a largo plazo, en general se asocia a la presencia de restos de cultivos en el campo que hacen que el suelo sea más rico en nutrientes o a la capacidad que tiene *R. solanacearum* de producir infecciones latentes colonizando las raíces de malezas que permanecen asintomáticas (Coutinho, 2005; Elphinstone, 1996; Janse et al., 2004; Pradhanang et al., 2000a). Después de multiplicarse en estos sitios protegidos, el patógeno vuelve al suelo iniciando un nuevo ciclo.

La supervivencia de *R. solanacearum* por largos períodos en el suelo y en agua también se atribuye a su capacidad de ingresar en un estado de dormancia denominado como "viable pero no cultivable" (Grey y Steck, 2001; Caruso et al., 2005). Muchos microorganismos de suelo son capaces de adoptar esta condición, sin embargo no se han determinado las causas que provocan este comportamiento ni las condiciones que favorecen su reversión.

Luego de lograr sobrevivir y diseminarse en el ambiente, la próxima etapa en el ciclo de vida de *R. solanacearum* es la de invadir a un hospedero susceptible. La colonización de la planta ocurre generalmente a través de heridas radiculares o por los sitios de emergencia de las raíces laterales. No se conoce exactamente el mecanismo involucrado en esta etapa de adherencia y reconocimiento del hospedero, sin embargo, se sugiere que la movilidad mediada por flagelo y la movilidad del tipo "*twitching*" mediada por fimbrias de tipo IV, juegan un rol importante en estos procesos (Kang et al., 2002; Liu et al., 2001; Tans-Kersten et al., 2001). También se ha comprobado que los exudados radiculares producen un efecto qumiotáctico sobre células de *R. solanacearum*, promoviendo la adherencia y eficiencia de colonización (Yao y Allen, 2006). Además, estos exudados inducen la expresión de genes cuyos productos pueden promover la invasión y colonización de la raíz y que actúan como factores tempranos de virulencia (Colburn-Clifford y Allen, 2010; Colburn-Clifford et al., 2010; Flores-Cruz y Allen, 2009). Estos factores permiten la supervivencia del patógeno en el entorno de la rizósfera y su adaptación al ambiente de la planta, teniendo un efecto positivo sobre la virulencia de *R. solanacearum.* Estos estudios sobre las etapas tempranas de virulencia en *R. solanacearum* se han realizado sobre plantas de tomate, y poco se conoce sobre lo que ocurre en otros hospederos.

Una vez dentro de la planta, *R. solanacearum* debe multiplicarse y transmitirse sistémicamente lo que lleva a la aparición de los síntomas de la enfermedad. El objetivo del patógeno en esta etapa es maximizar el tamaño de su población, para lo cual adapta su metabolismo a las condiciones existentes en el sistema vascular de su hospedero (Brown y Allen, 2004). La bacteria va progresivamente colonizando los espacios intracelulares de la corteza de la raíz y del parénquima vascular. Esto conduce a la ruptura de las paredes celulares facilitando su dispersión a través del sistema vascular dónde se reproduce rápidamente (Vasse, 1995). Aunque el xilema se describe a menudo como un ambiente pobre en nutrientes, se ha comprobado que *R. solanacearum* puede crecer muy bien en este medio alcanzando poblaciones que pueden llegar a títulos de 10^{10} ufc/g en una planta hospedera (Pegg, 1985; Schell, 1996). Los típicos síntomas de marchitamiento comienzan a evidenciarse a medida que aumenta la densidad del patógeno en el interior de la planta. En plantas de tomate, los primeros síntomas se correlacionan con una densidad bacteriana de más de 4 x 10^7 ufc/g de tejido a nivel del tallo (McGarvey et al., 1999). En esta etapa se producen altas cantidades de un polisacárido extracelular (EPS I) que interfiere con el transporte de agua a través de la planta (Denny y Baek, 1991; McGarvey et al., 1999). A medida que la población bacteriana aumenta el marchitamiento se hace generalizado pudiendo culminar con la muerte de la planta.

La marchitez bacteriana causada por *R. solanacearum*

R. solanacearum es el agente responsable de la *marchitez bacteriana*, una de las enfermedades de plantas más destructivas a nivel mundial que afecta a más de 200 especies distribuidas en 50 familias diferentes (Elphinstone 2005; Hayward 1994b). La familia Solanaceae es la más susceptible según el número de especies afectadas, causando importantes pérdidas económicas en cultivos como papa, tomate, morrón, berenjena, y tabaco. Otros hospederos incluyen, geranio, jengibre, maní, banana, plátano, mora, y varias especies de malezas.

La descripción de la enfermedad y las estrategias de control van a estar centradas en la papa como hospedero, por tratarse del cultivo principalmente afectado en Uruguay sobre el que se desarrolla esta tesis.

La marchitez bacteriana de la papa

En el cultivo de papa (*Solanum tuberosum* L.), la "marchitez bacteriana" también es conocida como "murchera" o "podredumbre parda" y constituye la enfermedad de origen bacteriano más importante, produciendo daños sobre el follaje y los tubérculos, restringiendo la producción y pudiendo ocasionar importantes pérdidas económicas (Allen et al., 2001; Elphinstone, 2005). El primer síntoma provocado es un marchitamiento de las hojas superiores las cuales adquieren progresivamente una coloración bronceada. Luego, se observa un marchitamiento generalizado que puede eventualmente culminar con la muerte de la planta. Al cortar el tallo, se observan exudados en la zona vascular y en el tubérculo aparece una coloración parda del anillo vascular y de los tejidos próximos, principalmente en la zona cercana al estolón. Es frecuente que aparezcan exudados blanquecinos y cremosos en esta zona, sin necesidad de presionar el tubérculo. Los síntomas exteriores pueden no ser visibles y en ocasiones se confunden con los producidos por otros patógenos. Al evolucionar la enfermedad se produce una necrosis del anillo vascular y de los tejidos adyacentes. La entrada de patógenos secundarios contribuye a que se produzca una pudrición total del tubérculo (Thurston, 1963).

Las cepas de *R. solanacearum* que afectan al cultivo de papa pueden pertenecer a los biovares 1, 2, 2-T, 3 y 4. Sin embargo, generalmente, en climas fríos y templados esta enfermedad se asocia a cepas del biovar 2, actualmente clasificadas como filotipo II, secuevar 1 (Elphinstone, 2005; López y Biosca, 2005). Este tipo de cepas presenta un rango de hospederos acotado, afectando especialmente a plantas de la familia de las Solanáceas, principalmente papa y tomate (Hayward, 1991). Sin embargo, en los últimos años se han reportado nuevos hospederos incluyendo otras Solanáceas como morrón y berenjena así como plantas de otras familias como geranio, repollo, y varias especies de malezas (Álvarez et al., 2008b; Elphinstone, 2005; Pradhanang et al., 2000a).

Otro rasgo particular que caracteriza a este tipo de cepas es su capacidad de producir enfermedad en climas fríos a diferencia de otras cepas de *R. solanacearum* que están adaptadas a regiones cálidas y tropicales (Swanson et al., 2005). También se ha comprobado que estas cepas son capaces de persistir durante largos períodos de

tiempo en el suelo y en cursos de agua (Álvarez et al., 2008a; Van Elsas et al., 2000). Además pueden producir infecciones latentes a bajas temperaturas en cultivos como tomate, papa, geranio y una variedad de malezas, lo que tiene una gran relevancia epidemiológica. El patógeno puede alcanzar poblaciones muy altas (de hasta 10^8 ufc/g tejido vegetal) sin que la planta presente síntomas (Elphinstone et al., 1998; Swanson et al., 2005). Este fenómeno constituye una importante vía de diseminación de la enfermedad, cuando el cultivo se inicia a partir de material vegetativo infectado, como tubérculos de papa o plantines de geranio. Un ejemplo de esto, es la experiencia ocurrida en los últimos años en Estados Unidos, donde se han constatado varias introducciones de *R. solanacearum* filotipo II, secuevares 1-2 a partir de la importación de cortes de geranios infectados provenientes de Guatemala y Kenia (Williamson et al., 2002). Debido al riesgo potencial de propagación de este patógeno a los cultivos de papa en zonas templadas, se incluyó en la lista de agentes de la Ley Agrícola de 2002 sobre Bioterrorismo del Departamento de Agricultura de los Estados Unidos (USDA, 2004). Este tipo de cepas también es considerado una bacteria de cuarentena incluida en la lista A2 de la Unión Europea (Lambert, 2002). Se ha constatado la propagación de *R. solanacearum* a través de los cursos de agua, donde la bacteria es capaz de sobrevivir en distintas especies de plantas silvestres, destacando entre ellas *S. dulcamara*, una Solanácea muy abundante presente en las orillas de los ríos y que puede actuar como reservorio del patógeno (Elphinstone et al., 1998; Caruso et al., 2005). En los últimos años han aumentado el número de focos de esta enfermedad en varios países de Europa lo que ha llevado a reforzar los controles sobre este patógeno a los efectos de disminuir su diseminación (Marco-Noales et al., 2008).

Medidas de control de la enfermedad

La marchitez bacteriana es considerada una de las enfermedades más difíciles de controlar (Saddler, 2005). No existe una estrategia de control universal para esta enfermedad. Esto puede explicarse por los diferentes tipos de suelos, climas y condiciones ecológicas donde se desarrollan los cultivos de papa así como por la gran diversidad que caracteriza a las cepas de este patógeno (López y Biosca, 2005). La capacidad de sobrevivencia de *R. solanacearum* en el suelo o en cursos de agua y la

ocurrencia de infecciones latentes, contribuyen a mantener al patógeno en el ambiente dificultando el manejo de la enfermedad. Otro aspecto a considerar es la ausencia de un alto grado de resistencia genética estable en cultivares comerciales de papa y la dificultad de las especies tetraploides como la papa para incorporar genes de resistencia en programas de mejoramiento (Boshou, 2005).

Dada la importancia y complejidad de este patosistema, es fundamental tener un adecuado conocimiento de la ecología del patógeno y de la epidemiología de la enfermedad. La mejor estrategia para lograr el control efectivo de esta enfermedad es la prevención. Esto significa, que las medidas a tomar no pueden limitarse al período de cultivo, siendo esencial que muchas decisiones sean tomadas antes de la siembra, en total coherencia con la filosofía de manejo integrado (Saddler, 2005). La incidencia de la marchitez bacteriana sólo puede reducirse si se combinan diversos componentes de control: uso de semilla certificada, siembra en suelo libre de patógeno, desarrollo de variedades resistentes, rotación con cultivos no hospederos y la aplicación de diversas prácticas agrícolas de saneamiento. Un buen manejo integrado de esta enfermedad puede conducir a su reducción significativa e incluso a su erradicación (French, 1994).

Resistencia del hospedero

La introducción de resistencia a *R. solanacearum* en el hospedero constituye la estrategia más efectiva para alcanzar el control de la enfermedad a largo plazo (Boshou, 2005). Sumado a la aplicación de buenas prácticas de producción y de medidas preventivas, el contar con el respaldo de un genotipo con cierto grado de resistencia de campo contribuye a disminuir las pérdidas económicas brindando una mayor seguridad productiva (Fock et al., 2000).

Las especies silvestres relacionadas representan un recurso muy valioso para el mejoramiento genético de los cultivos y en el caso de la papa constituyen un potencial único de mejoramiento debido a dos razones fundamentales: i) existen aproximadamente 200 especies silvestres de papa representadas por seis niveles de ploidía que se distribuyen a lo largo de todo el continente americano; ii) la enorme variabilidad que caracteriza en general a estas especies hace que constituyan un

reservorio genético de valor incalculable por sus características de resistencia a plagas y enfermedades, así como por su adaptación a factores abióticos y climáticos (Estrada Ramos, 1995).

El banco genético de papa más grande del mundo es mantenido por el Centro Internacional de la Papa, en Perú. En él se mantienen más de 3800 papas tradicionales andinas cultivadas así como 1500 muestras de aproximadamente 100 especies silvestres recolectadas en ocho países de América Latina. Esta colección representa un respaldo a largo plazo que se opone a la preocupante pérdida de diversidad genética asociada a este cultivo (Huamán et al., 1997).

Las especies silvestres de papa se extienden desde el sur de Estados Unidos hasta el extremo sur de Chile. En cuanto a su distribución geográfica se pueden reconocer claramente dos centros de diversidad: uno en la región central de México y otro que abarca la región andina a través de Perú, Bolivia y el norte de Argentina. Algunas de estas especies silvestres presentan una zona de distribución amplia (*S. colombianum*, *S. acaule*, *S. chacoense*) sin embargo la mayoría se encuentran restringidas a una zona ecológica determinada (Hawkes, 1991).

A pesar del gran potencial para el mejoramiento que presenta el cultivo, se han encontrado relativamente pocas fuentes de resistencia a *R. solanacearum*. Entre las especies silvestres con cierto grado de resistencia se incluyen: *S. raphanifolium, S. chacoense*, *S. microdontum, S. sparsipilum*, *S. stenotomum, S. multidissectum* y *S. commersonii* (Boshou, 2005). También se ha observado resistencia en accesiones de la especie cultivada diploide *S. phureja* pero la respuesta resultó dependiente de la temperatura y solo efectiva en climas fríos (French y De Lindo, 1982). La gran variabilidad genética que caracteriza a *R. solanacearum* y la influencia de las condiciones ambientales sobre la respuesta de defensa de la planta constituyen las principales dificultades en la obtención de cultivares con resistencia duradera (Anguiz y Mendoza, 1997). Hasta el momento se han desarrollado algunos cultivares tolerantes: "Achatt", de origen alemán que posee en su genealogía germoplasma de especies de *Solanum* silvestre (*S. acaule y S. stoloniferum)*, y cultivares derivados del germoplasma del Centro Internacional de la Papa (CIP) como el cultivar "Molinera" (Boshou, 2005). Otro factor que se debe tener en cuenta es el riesgo de usar variedades tolerantes o

con baja susceptibilidad ya que pueden enmascarar la presencia de la bacteria mediante infecciones asintomáticas. Estos hallazgos revelaron la necesidad de implementar criterios más exigentes de selección en los programas de mejoramiento que incluyan la detección de infección latente, de forma de evitar la generación de variedades tolerantes que promuevan la diseminación de la enfermedad en condiciones climáticas predisponentes (Priou et al., 2001, Priou et al., 2005).

Detección y diagnóstico de *R. solanacearum*

El diagnóstico precoz de *R. solanacearum* juega un papel fundamental en varias de las medidas que involucra el control integrado de la enfermedad. Los tubérculos-semilla infectados constituyen una de las principales vías de diseminación de *R. solanacearum* (Hayward, 1991). Principalmente en climas fríos y templados, las plantas infectadas pueden no mostrar síntomas, sin embargo pueden albergar a la bacteria y transmitirla a los tubérculos en los cuales se mantienen en forma latente, produciendo severos brotes de la enfermedad cuando son sembrados en condiciones más favorables (Ciampi y Sequeira, 1980; Hayward, 1991). Por lo tanto, es imprescindible, en forma previa al cultivo, certificar la ausencia del patógeno en la papa-semilla. Otras estrategias de control recomendadas incluyen el uso de campos libres de patógeno, la detección prematura de plantas infectadas en los cultivos y el monitoreo de las fuentes de agua de irrigación (López y Biosca, 2005; Priou et al., 2010). Para el éxito de todas estas medidas se requiere de sistemas de diagnóstico confiables y suficientemente sensibles, que contribuyan a evitar la diseminación y propagación de la enfermedad en el cultivo.

Varios organismos internacionales como USDA (*United States Department of Agriculture*) y EPPO (*European and Mediterranean Plant Protection Organization*) han elaborado protocolos de diagnóstico de *R. solanacearum* (Anonymous, 2006; Floyd, 2008). Los métodos recomendados incluyen: bioensayos, cultivo en medios semiselectivos, técnicas serológicas y moleculares. Cada método presenta sus ventajas y limitaciones en lo que refiere a límite de detección, especificidad, rapidez, aplicabilidad y costos. En general, la mejor estrategia a seguir implica el uso simultáneo

de varios métodos, a los efectos de lograr un diagnóstico confiable (Denny, 2006; Elphinstone et al., 1996).

Métodos tradicionales

La detección de *R. solanacearum* a partir de plantas con síntomas es relativamente sencilla, ya que en esta etapa el patógeno se encuentra en poblaciones elevadas $>10^8$ ufc por gramo de tejido. El típico exudado bacteriano puede observarse directamente al cortar un tubérculo, o a través de una simple prueba de flujo a partir de un corte del tallo de la planta (Allen et al., 2001). Este exudado puede considerarse prácticamente como un cultivo puro de la bacteria, por lo que resulta fácil lograr su aislamiento en un medio de cultivo general para luego proceder a su identificación. La detección de *R. solanacearum* en suelo o agua es más difícil ya que el patógeno se encuentra en bajo número y por la presencia de otros microorganismos saprófitos que se encuentran en mayor proporción o pueden desarrollarse más rápido (Denny, 2006). Para el aislamiento a partir de estas muestras existen medios de cultivo específicos que favorecen la recuperación de *R. solanacearum* en cultivo, aunque el resultado que se obtiene es variable y dependiente de las condiciones de cada muestra (Elphinstone et al., 1996; Imazaki y Nakaho, 2010).

Métodos serológicos

Los ensayos serológicos se utilizan todavía como métodos de tamizaje en muchos laboratorios, ya que son relativamente sencillos, rápidos y de bajo costo. Estos métodos pueden estar asociados a problemas de especificidad o sensibilidad, según el tipo de anticuerpos utilizado (Denny, 2006). En general, los anticuerpos policlonales presentan mejor sensibilidad porque suelen reaccionar con más fuerza con las células de *R. solanacearum* que los anticuerpos monoclonales. Sin embargo, esta mayor sensibilidad se ve compensada por la reducción de la especificidad debido a la ocurrencia de una tasa alta de falsos positivos, principalmente en presencia de contaminantes en la muestra que promueven reacciones cruzadas (Griep et al., 1998).
El método de ELISA (*Enzime Linked Inmuno Assay*) es el que mayor aplicación tiene, y se han desarrollado ensayos con diferentes variantes, que se encuentran disponibles

comercialmente. En el Centro Internacional de la Papa, se desarrolló un método de ELISA para la detección de *R. solanacearum* en tubérculos con infección latente (Priou et al., 1999; 2001). El método incorpora una etapa de enriquecimiento previo al inmunoensayo que permite mejorar la sensibilidad de detección al aumentar el número de células blanco. Otra variante que se introduce es la de realizar el ensayo fijando la muestra a una membrana de nitrocelulosa, la cual se puede conservar intacta por tiempo indefinido. Posteriormente, se desarrolló un ensayo de ELISA-*sandwich* (DAS-ELISA) que introduce el uso de un anticuerpo monoclonal para mejorar la especificidad del método (Caruso et al., 2002). Este método también se aplicó luego de una etapa de enriquecimiento para la detección de infección latente en muestras de tubérculos. Los métodos serológicos también se han aplicado para la detección de *R. solanacearum* en otro tipo de muestras, incluyendo suelo y tallos de plantas asintomáticas (Priou et al., 2006; 2010).

Métodos basados en ADN

Los métodos de detección basados en PCR generalmente presentan mayor sensibilidad, especificidad y requieren menor tiempo de análisis. Estos métodos actualmente han sido incorporados como métodos de rutina en la mayoría de los laboratorios de diagnóstico de los países desarrollados. Sin embargo, en muchos países aún no se ha logrado introducir esta tecnología debido principalmente a restricciones de equipamiento y capacitación. En 1992 fue reportado el primer método de detección de *R. solanacearum* por PCR (Seal et al., 1992). A partir de entonces, han surgido diversas variantes dirigidas a lograr una mayor sensibilidad, especificidad y aplicabilidad sobre distintas muestras ambientales.

Los métodos descritos utilizan *primers* dirigidos a diversos blancos de detección, incluyendo el gen del 16S rDNA, la región intergénica (ITS) en el operón rRNA y varios genes funcionales (Boudazin et al., 1999.; Pastrik y Maiss, 2000; Pastrik et al., 2002; Poussier y Luisseti, 2000; Poussier et al., 2000; Schonfeld et al., 2003 Seal et al., 1993; Seal et al., 1999). Otro aspecto a considerar en la elección del sistema de *primers* es hacia que grupo de cepas está dirigida la reacción. Muchos sistemas apuntan a detectar a todas las cepas de *R. solanacearum* (Opina et al., 1997; Pastrick y Maiss,

2000; Seal et al., 1993). Sin embargo, dada la diversidad que presenta este patógeno, se ha visto que algunas de estas reacciones tienen baja especificidad lo que introduce el riesgo de que ocurran falsos negativos o falsos positivos de detección (Arahal et al., 2004). Por otro lado, se han diseñado reacciones de PCR dirigidas a detectar grupos específicos de cepas de *R. solanacearum* (Fegan et al., 1998; Pastrik et al*.,* 2002; Poussier y Luisetti, 2000; Seal et al., 1999). Otro ejemplo de este tipo es el método desarrollado para la determinación de los filotipos establecidos en el nuevo sistema de clasificación (Fegan y Prior, 2005). Este método está basado en una reacción de multiplex-PCR que consiste en una variante del método clásico de PCR, en la cual se amplifican simultáneamente dos o más *loci* en una misma reacción. En este caso, se obtiene un producto de amplificación específico para cada uno de los filotipos (basado en la secuencia ITS) y un producto común para todas las cepas de la especie (*primers* 759/760, Opina et al*.,* 1997). De esta forma, aplicando una única reacción de PCR es posible avanzar en la identificación y caracterización de las cepas aisladas.

La presencia de sustancias inhibidoras en las muestras de suelo, agua, o extractos vegetales, es quizás el mayor problema que limita el uso de la PCR como método de diagnóstico (Denny, 2006). Una de las estrategias más utilizada para solucionar este problema consiste en el agregado de una etapa de enriquecimiento de la muestra en un medio selectivo previo a la reacción de PCR (Lin et al., 2009; Pradhanang et al., 2000b). Por medio de este procedimiento, comúnmente denominado BIO-PCR, se logra disminuir la concentración de las sustancias inhibidoras al mismo tiempo que se aumenta el número de células diana, mejorando la sensibilidad de detección. Además, se han descrito diversos protocolos de extracción de ADN así como el agregado de aditivos para la reacción de PCR que contribuyen a evitar el efecto de las sustancias inhibidoras (Poussier et al., 2002). Otra estrategia utilizada, es el uso de un control interno de amplificación, mediante la incorporación de *primers* que co-amplifican un producto que se encuentre presente (o se agrega) en todas las muestras (ej. *primers* que amplifican un gen de la planta hospedera) (Pastrik et al., 2002). De esta forma, el método permite identificar falsos negativos atribuidos a bajos rendimientos en el aislamiento del ADN o a la presencia de sustancias inhibidoras de la PCR.

Por otro lado, también se han desarrollado varias modificaciones de la reacción de PCR convencional, dirigidas a mejorar la sensibilidad de las técnicas de detección. Una de las variantes introducidas consiste en la aplicación de una reacción de *Nested-PCR* o PCR anidada, la cual comprende dos rondas de amplificación con distintos pares de *primers* en cada una (Poussier y Luissetti, 2000; Pradhanang et al., 2000b). Sin embargo, esta técnica también posee algunas desventajas, como el riesgo de contaminación asociado a la manipulación de material amplificado en la primera etapa y el mayor tiempo y costos necesarios debido a la realización de dos rondas de PCR (Denny, 2006). Otra alternativa, consiste en la aplicación de un método de Co-PCR basado en la acción simultánea de tres *primers* en una única reacción de PCR. Este método ha sido utilizado con éxito en la detección de *R. solanacearum* en cursos de agua (Caruso et al., 2003). En los últimos años también se han desarrollado métodos de detección por PCR en tiempo real (Huang et al., 2009; Ozakman y Schaad, 2003; Smith y Boher, 2009; Weller et al., 2000). Esta tecnología se ha aplicado a la detección de *R. solanacearum* en muestras de tubérculos, tallo y suelo, logrando mejorar la sensibilidad de detección. Además, este método reduce los tiempos de análisis ya que no requiere análisis posterior a la amplificación y permite cuantificar el ADN diana. Sin embargo, los costos asociados al equipamiento e insumos necesarios hacen que su uso todavía sea limitado, principalmente en los países en desarrollo.

Mecanismos de patogenia

R. solanacearum posee múltiples factores de virulencia que actúan coordinadamente en diferentes etapas a lo largo del proceso de patogenia sobre un hospedero susceptible. Estas etapas no son específicas sino que describen en general los procesos infecciosos que ocurren en plantas, animales o humanos y se pueden resumir en: i) contacto o entrada del patógeno en el hospedero, ii) evasión de mecanismos de defensa del hospedero y multiplicación del patógeno, iii) producción del daño sobre el hospedero, aparición de síntomas de la enfermedad, iv) diseminación y dispersión del patógeno. Los mecanismos que intervienen en la patogenia de *R. solanacearum* han sido estudiados intensamente en los últimos 50 años y varios autores han publicado revisiones sobre este tema (Denny, 2005; Genin, 2010; Genin y Boucher, 2004;

Poueymiro y Genin, 2009; Schell, 2000). A continuación se resumen los principales factores de virulencia identificados hasta el momento.

Polisacárido extracelular

El polisacárido extracelular ácido (EPS I) es considerado uno de los principales factores de virulencia de *R.* solanacearum. Se trata de un largo polímero de peso molecular mayor a 10^6 Da que consiste en unidades repetidas formadas por tres monosacáridos inusuales: N-acetil-galactosamina, ácido 2-N-acetil-2-desoxi-L-galacturónico, y 2-N-acetil-4-N-(3-hydroxybutanoyl)-2,4,6-trideoxy-D-glucosa (Orgambide et al., 1991). Este complejo polisacárido es producido por *R. solanacearum* en grandes cantidades en varios medios de cultivo y en el interior de la planta (Araud-Razou et al., 1998; McGarvey et al., 1998). EPS I es producido por todas las cepas de *R. solanacearum* y contribuye a la virulencia al bloquear físicamente los vasos de xilema obstaculizando el transporte de agua a través de la planta (Denny et al., 1988; Denny et al., 1990; Kao et al., 1992; McGarvey et al., 1999). Las cepas de *R. solanacearum* mutantes para la producción de EPS I no son capaces de producir síntomas de marchitamiento, incluso cuando son inoculadas directamente en el tallo de la planta (McGarvey et al., 1999; Araud-Razou et al., 1998; Saile et al., 1997). Se ha encontrado que el EPS I también promueve la colonización del sistema vascular de la planta a través de las raíces, posiblemente enmascarando la superficie expuesta del patógeno evitando así el reconocimiento por el sistema de defensa de la planta (McGarvey et al., 1999; Saile et al., 1997; Schell 2000).

Movilidad

Tanto la movilidad mediada por flagelo como la movilidad del tipo "twitching" mediada por fimbrias de tipo IV, juegan un rol importante en la virulencia de *R. solanacearum* (Kang et al., 2002; Liu et al., 2001; Tans-Kersten et al., 2001; Tans-Kersten et al., 2004).

En cepas mutantes no flageladas debido a la inactivación del gen estructural de la flagelina (FliC) o del interruptor del motor flagelar (FliM), se observa una reducción de la virulencia cuando se utiliza un método de inoculación en suelo. Por el contrario, la

virulencia no se ve afectada cuando las cepas son inoculadas directamente en el tallo de plantas de tomate (Tans-Kersten et al., 2001). Además, también se comprobó que la cepa salvaje no presenta flagelo cuando se encuentra en el interior de las plantas de tomate, lo que sugiere que la movilidad mediada por flagelo contribuye específicamente en las etapas tempranas de colonización pero no es requerida una vez que la bacteria se encuentra en el interior de la planta (Tans-Kersten et al., 2001; Tans-Kersten et al., 2004).

Las bacterias móviles pueden presentar movimientos de atracción o repulsión determinados por diferentes estímulos. En *R. solanacearum* se ha comprobado que los exudados radiculares producen un efecto qumiotáctico sobre las células que se encuentran en la rizósfera, lo que promueve la adherencia y eficiencia de colonización (Yao y Allen, 2006). Esto sugiere que el movimiento dirigido por señales químicas emanadas de las raíces de las plantas hospederas es importante para que se desarrolle una virulencia completa, y no simplemente el movimiento al azar mediado por flagelos (Yao y Allen, 2006).

Otro tipo de movilidad que se comprobó que tiene un efecto sobre la virulencia de *R. solanacearum* es la movilidad tipo "twitching", la cual es un mecanismo de translocación sobre superficies caracterizada por tirones cortos e intermitentes y que está mediada por la retracción de fimbrias o pili de tipo IV (Mattick, 2002). Las fimbrias de tipo IV son estructuras superficiales ampliamente difundidas entre bacterias que intervienen en varias funciones celulares además de la movilidad tipo *twitching*, entre los que se incluyen la adhesión a superficies, formación de biofilm y captación de ADN foráneo (Pelicic, 2008). También se ha reportado que estas estructuras intervienen en la fijación sobre los tejidos del hospedero, y son considerados importantes factores de virulencia en muchas bacterias patógenas (Pizzarro-Cerdá y Cossart, 2006). En *R. solanacearum* se descubrió la presencia de fimbrias de tipo IV, gracias a la observación de un fenotipo de colonia característico de cepas con movimiento tipo "twitching" (Liu et al., 2001). También se comprobó que cepas de *R. solanacearum* mutantes en algunos de los genes principales vinculados a la biogénesis y funcionamiento de las fimbrias de tipo IV mostraron una marcada reducción de la virulencia sobre plantas de tomate (Kang et al., 2002; Liu et al., 2001). Este efecto se observó mediante

inoculación directa del patógeno en el tallo de la planta y por inoculación en el suelo, por lo que se postula que estas estructuras superficiales juegan un rol en varias etapas del desarrollo de la enfermedad (Kang et al., 2002; Liu et al., 2001). Estas cepas mutantes también mostraron un fenotipo alterado en varias funciones mediadas por las fimbrias de tipo IV, por lo que con los experimentos realizados no se pudo concluir si la reducción de la virulencia en tomate se debe exclusivamente a la pérdida de movilidad tipo *twitching* (Kang et al., 2002).

Enzimas que degradan los componentes estructurales de la pared vegetal

R. solanacearum produce un conjunto de enzimas hidrolíticas extracelulares, que pueden intervenir en la degradación de los tejidos vegetales observada en etapas avanzadas de la enfermedad. Se han identificado seis enzimas extracelulares: una β-1,4-endoglucanasa (Egl), una exoglucanasa (ChbA), una endopoligalacturonasa (PehA o PglA), dos exopoligalacturonasas (PehB y PehC), y un pectinmetilestearasa (Pme) (Gabriel et al., 2006; González y Allen, 2003; Kang et al., 1994; Liu et al., 2005). Se postula que estas enzimas pueden facilitar la invasión de las raíces, la colonización, y la ruptura de los tejidos del hospedero. Sin embargo se ha demostrado, mediante inactivación de los genes individuales, que ninguna de estas exoenzimas es esencial para el desarrollo de la enfermedad y que su contribución a la virulencia de *R. solanacearum* es cuantitativa y variable entre las distintas cepas analizadas (Denny et al., 1990; González y Allen, 2003; Huang y Allen, 1997; Tans-Kersten et al., 1998).

En *R. solanacearum* la mayoría de estas enzimas y muchas otras proteínas extracelulares atraviesan la membrana interna a través de la vía de exportación Sec-dependiente y la membrana externa a través del sistema de secreción de tipo II (T2SS) (Preston et al., 2005; Schell, 2000). En tomate, la inactivación del T2SS produce un efecto más drástico sobre la virulencia de *R. solanacearum* que el observado para una cepa mutante en las seis enzimas degradadoras de la pared vegetal (Liu et al., 2005). Esto sugiere que existen otras proteínas aún no identificadas que son secretados por esta vía y que contribuyen a la virulencia de este patógeno (Liu y Denny, 2007; Poueymiro y Genin, 2009).

Sistema de secreción de tipo III y proteínas efectoras

Al igual que muchas bacterias patógenas Gram-negativas, *R. solanacearum* posee un sistema de secreción de tipo III que juega un rol esencial en su patogenia. Este sistema especializado de secreción actúa translocando directamente en el interior de la célula hospedera un conjunto de proteínas efectoras que modulan las funciones celulares del hospedero y alteran los sistemas de defensa de la planta (Alfano y Collmer, 2004). Este sistema es codificado por un *cluster* genético de 23-kb denominado *hrp,* que es esencial para la virulencia del patógeno sobre hospederos susceptibles así como para producir la reacción de hipersensibilidad en plantas no hospederas (Genin y Boucher, 2002). Mediante estudios citológicos se ha demostrado que este sistema es importante para la colonización de las raíces, ya que las cepas mutantes *hrp* son menos invasivas y alcanzan menores niveles de población en comparación con los observados para la cepa salvaje (Vailleau et al., 2007; Vasse et al., 2000).

A pesar de la intensa investigación en este tema y el rol central que tiene el sistema de secreción de tipo III en la virulencia de *R. solanacearum*, aún no se ha logrado establecer el modo de acción específico de las proteínas efectoras. Generalmente, estas proteínas tienen funciones bioquímicas sutiles pudiendo actuar de forma sinérgica y redundante entre sí, por lo que resulta difícil demostrar el rol que cumple cada efector individual en la patogenia (Denny, 2006). Se han aplicado diferentes estrategias para identificar el repertorio de proteínas efectoras en *R. solanacearum*, generalmente basados en la disponibilidad del genoma completo de varias cepas y en el conocimiento sobre la regulación transcripcional de este sistema (Cunnac et al., 2004; Gabriel et al., 2006; Mukaihara et al. 2004; Mukahiara y Tamura, 2009; Occhialini et al. 2005; Salanoubat et al. 2002). En la actualidad se han identificado más de 90 proteínas efectoras potencialmente secretadas por el sistema de secreción de tipo III, de las cuales aproximadamente una tercera parte se ha comprobado que realmente son translocadas al medio extracelular o al interior de la célula vegetal (Mujahiara y Tamura, 2009; Poueymiro y Genin, 2009).

Algunos de las proteínas efectoras se han identificado como proteínas de avirulencia, las cuales son reconocidas específicamente por receptores en la planta hospedera, desencadenando una respuesta de defensa de la planta en un tipo de interacción “gen

a gen" que es bastante infrecuente en *R. solanacearum* (Keen, 1990). AvrA es una proteína que actúa induciendo una respuesta de hipersensibilidad en varias especies de tabaco (Carney y Denny, 1990; Robertson et al., 2004). Por otro lado, el reconocimiento de las proteínas PopP1 y PopP2 está asociado a la resistencia específica en ciertos ecotipos de *Petunia* y *Arabidopsis* respectivamente (Deslandes et al., 2003; Lavie et al., 2002).

Una característica destacable de este repertorio de proteínas efectoras es la existencia de varias familias de genes, que están ampliamente conservadas dentro de las diferentes cepas de la especie (Poueymiro y Genin, 2009). Un ejemplo es la familia de efectores GALA, compuesta por proteínas con dominios LRR F-box, que se postula que interfieren en los procesos de ubiquitinación del hospedero (Angot et al., 2006). Esta vía se encuentra implicada en el recambio intracelular de las proteínas y juega un papel importante en la degradación de proteínas reguladoras de vida corta en organismos eucariotas (Hershko y Ciechanover, 1998). No se conocen las proteínas específicas sobre las cuales podrían actuar los efectores del tipo GALA, pero se postula que podrían tratarse de proteínas claves involucradas en los mecanismos de defensa de la planta. Por otra parte, estos efectores podrían dirigir la degradación de otras proteínas efectoras una vez que cumplieron su función, ya que el reciclado de estos efectores puede ser un punto crítico del proceso de patogenia (Poueymiro y Genin, 2009).

Otra observación general en el repertorio de proteínas efectoras es la presencia frecuente de repeticiones internas que están presuntamente implicadas en interacciones proteína-proteína: repeticiones tipo ankirina, diferentes tipos de repeticiones ricas en leucina (LRR) o nuevas estructuras como la familia de efectores SKWP (Poueymiro y Genin, 2009). Estos dominios son semejantes a los presentes en proteínas de células eucariotas, lo que sugiere que podrían interactuar con proteínas blanco en la célula hospedera, probablemente promoviendo la liberación de nutrientes o inhibiendo las respuestas de defensa de la planta (Alfano y Collmer, 2004)

Reguladores del crecimiento vegetal

La alteración de los mecanismos de defensa de la planta también involucra a moléculas bacterianas no proteicas que probablemente complementan la acción de las proteínas efectoras secretadas. *R. solanacearum* produce fitohormonas como el etileno, cuyas vías de señalización juegan un papel fundamental en el desarrollo de síntomas marchitamiento (Hirsch et al., 2002; Valls et al., 2006). Se comprobó que la producción de etileno en *R. solanacearum* está coordinada con la expresión del sistema de secreción de tipo III y que los niveles de etileno producidos son capaces de modular la expresión de genes del hospedero y por lo tanto interferir en los procesos de señalización asociados con los mecanismos de defensa de la planta (Valls et al., 2006). También se demostró que la producción de una molécula derivada del indol estaba bajo el control del regulador HrpB (Delaspre et al., 2007). Esta molécula, denominada factor difusible HrpB-dependiente (HDF) parece intervenir en el sistema de *quorum sensing*. Sin embargo, recientemente se ha demostrado que compuestos muy relacionados al HDF pueden inducir una respuesta de resistencia en la planta (Li et al., 2008).

Regulación de la expresión de los factores de virulencia en *R. solanacearum*

Durante su ciclo de vida *R. solanacearum* coloniza dos nichos ecológicos bien diferentes: el suelo, dónde los nutrientes son escasos y la competencia con otros microorganismos es intensa; y el interior de la planta hospedera dónde los nutrientes son relativamente abundantes pero están protegidos. Para poder adaptarse a ambos tipos de vida, *R. solanacearum* ha desarrollado un complejo sistema de regulación que responde a señales ambientales y que promueve cambios drásticos a nivel fisiológico (Denny, 2006; Schell, 2000).

Conversión fenotípica

La conversión fenotípica juega un rol central en las complejas redes de regulación que controlan la patogenia en *R. solanacearum*. El descubrimiento de este fenómeno estuvo asociado al estudio de mutantes espontáneos que normalmente aparecen durante el crecimiento de *R. solanacearum* en cultivo o su almacenamiento a largo

plazo (Kelman, 1954). Estos mutantes presentan varias alteraciones fenotípicas respecto a la cepa salvaje, incluyendo cambios en la morfología de colonia, pérdida de virulencia, reducción en la producción de EPS y alteración de los niveles de varias enzimas extracelulares (Allen et al., 1991; Denny et al., 1988; Kelman, 1954). Mediante estudios genéticos se descubrió que estos cambios estaban asociados a la mutación espontánea del locus PhcA, un regulador transcripcional que es un componente clave en la supervivencia y patogenia de *R. solanacearum,* y que actúa en respuesta a la disponibilidad de nutrientes y a la densidad celular (Brumbley et al., 1993; Schell, 2000).

Entre los factores que son regulados positivamente por PhcA se incluyen: la producción de EPS I, producción de algunas enzimas extracelulares (Egl, Pme), un sistema de *quorum sensing* mediado por homoserin-lactona, y la competencia natural para la transformación (Kang et al, 1994; Schell, 2000). PhcA también regula negativamente varios procesos entre los que se incluyen: producción de algunas enzimas extracelulares (PehA y PglA); biosíntesis de sideróforos; producción de fimbrias de tipo IV afectando la movilidad tipo *twitching*, auto agregación y la formación de biofilm; movilidad flagelar a alta densidad celular; actividad del regulador transcripcional HrpG (Bhatt y Denny, 2004; Genin et al., 2005; Schell 2000).

La expresión de PhcA está a su vez regulada por el sistema sensor de confinamiento codificado por el operón *phcBSR* (Clough et al., 1997). PhcB es una metiltransferasa requerida para la síntesis del 3-hidroxi metil éster del ácido palmítico (3-OH PAME), una molécula señal del sistema de *quórum sensing* que se acumula en el espacio extracelular cuando la bacteria se está multiplicando rápidamente en un lugar confinado (Flavier et al., 1997). PhcS y PhcR forman un sistema regulador de dos componentes que detecta los niveles de 3-OH PAME y responde aumentando el nivel de PhcA en la célula (Clough et al., 1997). En otras palabras, cuando existe baja densidad de población, las células tienen niveles bajos de PhcA, y al igual que los mutantes espontáneos en *phcA*, exhiben un fenotipo de baja virulencia. Se postula que este es el estado fisiológico que adoptan las células para lograr sobrevivir en el ambiente e invadir los tejidos vegetales, ya que tanto en el suelo como en el entorno de las raíces existen bajas densidades de células de *R. solanacearum* (Denny, 2005).

Una vez dentro del xilema, se alcanzan altas densidades de células lo que aumenta el nivel de PhcA. Esto desencadena la expresión de varios factores de virulencia que contribuyen al desarrollo de la enfermedad, y al mismo tiempo la represión de otros factores necesarios para las etapas tempranas de colonización.

Regulación del sistema de secreción de tipo III

En la mayoría de los sistemas estudiados, la transcripción del sistema de secreción de tipo III así como de la mayoría de las proteínas efectoras está controlada por una cascada de reguladores que actúan consecutivamente y en forma controlada. En *R. solanacearum* se sabe que este sistema está regulado por HrpB, un activador transcripcional de la familia AraC que se encuentra codificado dentro del cluster *hrp* (Genin et al., 1992). La inducción de HrpB está mediada por una cascada de regulación que se inicia con PrhA, una proteína de membrana externa que actúa como receptor de una señal no difusible específica de la planta hospedera (Aldon et al., 2000). La cascada de señalización desencadenada por el contacto con las células vegetales involucra a las proteínas PrhR, Prhl, PrhJ y HrpG (Aldon et al., 2000; Marenda et al., 1998; Schell, 2000). El regulón *hrp* es regulado negativamente por la represión de HrpG mediada por PhcA (Genin et al., 2005). Además, HrpG responde a señales provenientes de la planta así como a condiciones ambientales de estrés por falta de nutrientes, por lo que se postula la existencia de vías alternativas para la inducción de genes *hrp* en *R. solanacearum* (Denny, 2006).

Otros sistemas de regulación

Otros sistemas de regulación de dos componentes intervienen en el control de la expresión génica en *R. solanacearum.* Estos sistemas actúan en conexión con los sistemas ya descritos conformando una compleja red de regulación. (Allen et al., 1997; Schell et al., 1993).

Los sistemas VsrAD y VsrBC intervienen en la producción de EPS I y de varias proteínas extracelulares y la inactivación de estos sistemas está asociada a la pérdida de virulencia en tomate (Huang et al., 1995; Schell et al., 1993). VsrAD actúa promoviendo la transcripción de *xpsR,* el cual es activado también por PhcA. XpsR es un factor

transcripcional que actúa en coordinación con VsrBC activando la máxima expresión de EPS I (Garg et al., 2000, Huang et al., 1995). El sistema VsrAD también está implicado en varios fenotipos adicionales, incluyendo la capacidad de multiplicación dentro de la planta, la formación del biofilm, la tolerancia al frío, la sensibilidad al peróxido de hidrógeno, la movilidad y la regulación positiva de HrpG y HrpB, quizás a través de estrés nutricional (Huang et al., 1995, Denny, 2006).

Otro sistema que interviene en esta compleja red de regulación es PehSR, y su inactivación también está asociada a una pérdida de virulencia (Allen et al., 1997; Kang et al., 2002; Tans-Kersten et al., 2004). PehSR regula positivamente la producción de PehA, la movilidad flagelar y la producción de fimbrias de tipo IV (Allen et al., 1997; Kang et al., 2002; Tans-Kersten et al., 2001). A su vez, PhcA inhibe la expresión de *pehR* cuando se alcanzan densidades de población alta en cultivo (Allen et al., 1997; Tans-Kersten et al., 2004). Se postula que los sistemas VsrAD, VsrBC y PehSR son activados por señales propias de la planta aunque estas todavía no se han identificado (Denny, 2006).

Estudio de *R. solanacearum* en la era genómica

En los últimos años ha aumentado de forma continua el número disponible de secuencias genómicas de una variedad de microorganismos lo que ha impulsado la era de la genómica microbiana. En el caso particular de *R. solanacearum* la secuenciación y anotación del genoma completo de la cepa GMI1000 (filotipo I, secuevar 18) en el año 2002 ha sido un logro importante que ha contribuido al conocimiento sobre este patógeno (Salanoubat et al., 2002).

El genoma de *R. solanacearum* (GMI1000) está compuesto por dos replicones circulares de 3.7 Mb y 2.1 Mb con un contenido promedio de G+C de 67%. El replicón más grande, que tiene un origen de replicación típico de los cromosomas bacterianos y lleva los genes de todas las funciones esenciales, se conoce como el cromosoma. El replicón más pequeño tiene un origen de replicación característica de plásmidos y se lo reconoce como megaplásmido. En este replicón se encuentran muchos de los genes asociados a la virulencia y con la adaptación a diversos ambientes, por lo que se concluye que el megaplásmido es una parte esencial presente en todas las cepas de *R.*

solanacearum. Esta conclusión también se ve apoyada por el hecho que algunos genes esenciales como los relacionados con la biosíntesis de aminoácidos y cofactores están distribuidos en ambos replicones. Además, muchos genes propios del cromosoma, se encuentran duplicados en el megaplásmido. Estas evidencias sugieren que ambos replicones han co-evolucionado en forma conjunta en esta bacteria (Genin and Boucher, 2004; Salanoubat et al., 2002). Otro hallazgo es la existencia de regiones que presentan un uso alternativo de codones (ACURs) y un sesgo importante en el contenido G+C respecto al resto del genoma. Se han detectado 91 ACURs de entre 3 y 20-kb que abarca más del 7% del genoma de GMI1000 (Genin y Boucher, 2004). Generalmente estas regiones están flanqueadas por secuencias de inserción, elementos transponibles o de fagos, lo que sugiere que fueron adquiridas de otras especies por transferencia horizontal. Esto pudo haber sido facilitado por la habilidad natural que presenta *R. solanacearum* para incorporar ADN foráneo desde el ambiente (Bertolla et al., 1997). Además, estas regiones pueden promover la ocurrencia de otros rearreglos genómicos como deleciones, inserciones y duplicaciones. La gran flexibilidad del genoma de *R. solanacearum* podría ser una de las causas de la gran diversidad genética existente en este complejo de especies (Salanoubat et al., 2002; Genin y Boucher, 2004).

La disponibilidad de esta información genómica también ha contribuido a generar conocimiento sobre la patogenia de *R. solanacearum*, ya que ofrece la posibilidad de aplicar nuevas estrategias para el descubrimiento de los determinantes que intervienen en la virulencia.

Dado el rol central que tiene el sistema de secreción de tipo III en la patogenia de *R. solanacearum* se ha aprovechado la información de secuencia disponible para la identificación de proteínas efectoras potencialmente secretadas por este sistema. Una primera aproximación se obtuvo aplicando un enfoque bioinformático integrando diferentes criterios que incluyen: i) búsqueda de homología con proteínas efectoras ya identificadas en otros agentes patógenos; ii) presencia de dominios característicos de proteínas eucariotas; iii) presencia de motivos específicos en los promotores que sugieran que su transcripción pueda estar co-regulada con los genes *hrp* (Cunnac et al., 2004; Genin y Boucher 2004). Una estrategia alternativa se aplicó a partir del

desarrollo de un chip de microarrays conteniendo todos los genes identificados en la cepa GMI1000. Este chip se utilizó para la identificación de los genes regulados por HrpB y HrpG, los principales moduladores de la expresión de este sistema de secreción en *R. solanacearum* (Occhialinni et al., 2005; Valls et al., 2006).

Otra estrategia adoptada para analizar los genes involucrados en la patogenia de *R. solanacearum* es la técnica conocida como IVET (*In Vivo Expression Technology*) (Osbourn et al., 1987). Esta técnica permite identificar promotores inducidos durante la interacción de un microorganismo patógeno con su hospedero. Esta estrategia fue aplicada para la identificación de los genes expresados por *R. solanacearum* durante su interacción en la rizósfera de plantas de tomate así como en el interior de la planta de tomate, en etapas más avanzadas de la enfermedad (Brown y Allen, 2004; Colburn-Clifford y Allen, 2010). Los genes identificados a partir de estos estudios reflejan el proceso de adaptación que sufre *R. solanacearum* para lograr colonizar y multiplicarse en el interior del hospedero, y están asociados a diversas funciones incluyendo la respuesta al estrés, metabolismo, transporte y secreción, regulación transcripcional, y patogenicidad (Brown y Allen, 2004; Colburn-Clifford y Allen, 2010; Colburn-Clifford et al., 2010; Flores-Cruz y Allen, 2009; Yao y Allen, 2006).

Actualmente, la información que surgió a partir del análisis del genoma de la cepa GMI1000 ha sido ampliada gracias a la secuenciación de 6 cepas adicionales de *R. solanacearum* pertenecientes a otros filotipos y aisladas de diferentes hospederos (Gabriel et al., 2006; Guidot et al., 2009; Remenant et al., 2010). La cepa GMI1000 es una cepa aislada a partir de tomate en la Guayana Francesa, que presenta un amplio rango de hospederos y pertenece al filotipo I, secuevar 18. Posteriormente, se secuenciaron tres cepas adicionales de *R. solanacearum* pertenecientes a filotipo II y que muestran una estrecha gama de hospederos: IPO1609/UW551 (filotipo IIB, secuevar 1), que afecta principalmente a papa en climas fríos y templados; y Molk2 (filotipo IIB, secuevar 3), que causa la enfermedad del Moko del banano y el plátano (Gabriel et al, 2006;. Guidot et al, 2009). Recientemente, se publicó la secuencia genómica de tres cepas adicionales lo que permitió contemplar al resto de los grupos presentes en el complejo de especies de *R. solanacearum.* En este último estudio se incluyeron tres cepas aisladas a partir de tomate pero de diferentes orígenes

geográficos: la cepa Americana CFBP2957 (filotipo IIA, secuevar 36), la cepa CMR15 de origen africano (filotipo III, secuevar 29) y por último una cepa de Indonesia PSI07 (filotipo IV, secuevar 10) (Remenant et al, 2010).

Esta información resulta esencial para poder realizar comparaciones a gran escala y generar conocimiento sobre los determinantes genéticos que intervienen en la patogenia y en la especificidad de hospederos, así como profundizar en los aspectos evolutivos y de diversificación de *R. solanacearum*.

Hibridación genómica comparativa basada en *microarrays*

La era genómica iniciada a partir de la secuenciación del genoma completo de microorganismos abrió las puertas de la investigación microbiológica molecular a escala genómica y del desarrollo de nuevas tecnologías, como es el caso de los *microarrays* de ADN (Ehrenreich, 2006). La primera aplicación para la que se ha usado esta tecnología ha sido la generación de perfiles transcripcionales, permitiendo monitorizar en un solo experimento la expresión de un genoma bacteriano completo (Hughes et al., 2000).

Otra aplicación importante que surge a partir del desarrollo de la tecnología de *microarrays* es la hibridación genómica comparativa (del inglés, CGH), que permite realizar comparaciones a nivel de homología de ADN entre genomas bacterianos completos. Esta técnica se basa en la comparación de dos muestras de ADN genómico: una obtenida a partir de la cepa objeto de análisis y la otra a partir de una o varias cepas de referencia, cuyo contenido genético se encuentra representado en el chip. Como resultado de esta aplicación, se puede establecer el contenido genético de una cepa problema en relación al genoma de referencia.

El uso de CGH ha permitido desarrollar un nuevo enfoque filogenómico comparativo que ha resultado muy útil para revelar y analizar la diversidad genética entre diferentes cepas de la misma especie (Dorrell et al., 2005). Varios ejemplos pioneros son los estudios realizados sobre las variaciones en el genoma para diferentes cepas de *Helicobacter pylori* (Salama et al. 2000) o *Streptococcus pneumoniae* (Hakenbeck et al., 2001). En el caso de *R. solanacearum,* esta herramienta se aplicó recientemente para comparar el contenido genético de varias cepas de *R. solanacearum* pertenecientes a

diferentes filotipos (Guidot et al., 2007). Este estudio permitió identificar un conjunto de “genes centrales” que son conservados entre todas las cepas de *R. solanacearum,* y un conjunto de genes variables, agrupados en islas genómicas y distribuidos tanto en el cromosoma como en el megaplásmido (Guidot et al., 2007).

Esta técnica también se ha utilizado para realizar comparaciones genómicas entre cepas de la misma especie con diferente origen o grado de patogenicidad, lo que ha contribuido a identificar genes necesarios para la virulencia o la adaptación a un nicho hospedador-específico (Hotopp et al., 2006; Sarkar et al., 2006; Sung et al., 2008).

ANTECEDENTES DE TRABAJO

Importancia de *R. solanacearum* en Uruguay

En Uruguay *R. solanacearum* afecta principalmente al cultivo de papa, uno de los principales cultivos hortícolas del país. La enfermedad constituye un factor limitante para el cultivo ocasionando importantes pérdidas cuando las medidas de control preventivo no son respetadas (DIEA - MGAP). Además, representa un factor limitante para la certificación de papa-semilla nacional, que tiene una exigencia de "tolerancia cero" para este patógeno (INASE). La incidencia de murchera en nuestro país es variable y se caracteriza por la ocurrencia periódica de brotes agudos muy difíciles de prevenir y controlar. El primer reporte oficial fue registrado en 1974 en el departamento de Rocha y el impacto fue devastador (Canale, 1984). En esta oportunidad, el brote afectó a un 59% de los productores causando pérdidas variables entre un 5% y un 90% de la producción (Canale, 1984; Verdier, 2009). Un segundo brote importante ocurrió en la zafra 2001-2002 cuando el 34% de los predios sembrados fueron afectados (DIEA – MGAP). Actualmente, la incidencia de la enfermedad es menor pero se han detectado brotes en todas las zonas de producción, lo que dificulta enormemente el mantenimiento de áreas libres de patógeno y la propia continuidad del cultivo.

Hasta el momento, se han realizado muy pocos trabajos enfocados al estudio de este patógeno en Uruguay. Un relevamiento llevado a cabo durante la década de 1980 reveló la presencia de cepas que fueron caracterizadas como raza 3, biovar 2 (Canale y Peralta, 1982). En el año 2003, luego del severo brote que afectó a una gran proporción de los productores, se reinició el trabajo en el tema y se aislaron nuevas cepas del patógeno. La identificación preliminar de estas cepas reveló que todas pertenecen al biovar 2, el cual se corresponde con el filotipo II, secuevar 1 (Siri, 2005).

Estrategias para el control de la enfermedad

En Uruguay se han iniciado varios estudios multidisciplinarios, enfocados al desarrollo de nuevas estrategias para el control integrado de la marchitez bacteriana en el cultivo de papa. Desde hace varios años se está trabajando en forma conjunta con los

diferentes actores involucrados en la producción del cultivo, con el objetivo de mejorar el manejo de esta enfermedad.

En la estación experimental Las Brujas del Instituto Nacional de Investigación Agropecuaria (INIA) se lleva a cabo el programa de mejoramiento genético de papa y desde el año 2000 se trabaja en la introducción de resistencia a la marchitez bacteriana en las nuevas variedades. La especie silvestre uruguaya más valiosa para el mejoramiento genético del cultivo de papa es *Solanum commersonii*. Esta especie está ampliamente distribuida en nuestro territorio, y presenta una importante diversidad morfológica y química (Vázquez et al., 1997). Su gran resistencia al frío y a algunas plagas ha despertado gran interés en la comunidad de mejoradores de papa. Sin embargo, su utilización ha sido limitada por barreras en la reproducción sexual con la papa (*S. tuberosum*: 4x, 4EBN) y con especies diploides (2x, 2EBN). Estudios realizados en la Universidad de Wisconsin han reportado resistencia a *R. solanacearum* en accesiones de *S. commersonii* (Bamberg et al., 1994) y también se han obtenido híbridos somáticos entre *S. commersonii* y *S. tuberosum* con importantes niveles de resistencia a este patógeno (Carputo et al., 2009; Laferriere et al., 1999). Sin embargo, estos estudios partieron de un número limitado de accesiones, no contemplándose el potencial de diversidad que tiene esta especie silvestre.

Uruguay constituye el centro de diversidad primario de *S. commersonii*, lo que facilita su disponibilidad y una gran variabilidad para su introducción al mejoramiento. Se realizó un relevamiento de germoplasma silvestre de *S. commersonii* presente en diferentes puntos del país y en la actualidad se cuenta con una colección que es conservada en el INIA. Se analizó la diversidad genética de la especie lo que permitió conocer la distribución de los distintos genotipos y contribuir a su aprovechamiento como fuente de germoplasma (Siri et al., 2009). Esta colección también fue caracterizada por su resistencia a *R. solanacearum* y otras características importantes (contenido de glicoalcaloides, ploidía, fertilidad, etc.) (Galván et al., 2007; Pianzzola et al., 2005; Siri, 2005; Siri et al. 2005; 2009). A partir de la información generada se seleccionaron las primeras accesiones para ser usadas como progenitores en los cruzamientos, que actualmente se encuentran en etapa de evaluación (González, 2010).

JUSTIFICACIÓN

En los últimos años, se han iniciado estudios enfocados al desarrollo de medidas de control de la marchitez bacteriana que afecta al cultivo de papa en Uruguay. Algunos de estos trabajos apuntaron al aprovechamiento de la especie silvestre *S. commersonii* como fuente de germoplasma para el desarrollo de variedades de papa resistentes. Sin embargo, la evaluación de resistencia y selección de genotipos en el programa de mejoramiento se realizaron utilizando una única cepa de *R. solanacearum*. Dada la gran variabilidad que puede presentar este patógeno, en cuanto a rango de hospederos, agresividad y características ecológicas, es necesario ampliar este estudio utilizando un mayor número de cepas locales abarcando las diferentes zonas de producción del cultivo de papa en Uruguay.

OBJETIVOS Y DESCRIPCIÓN DE LA TESIS

El objetivo general de esta tesis es generar conocimiento sobre las cepas de *R. solanacearum* que afectan los cultivos de papa en Uruguay, que permita avanzar en el desarrollo de estrategias para su control.
Para ello, se plantean los siguientes objetivos específicos:

1) Realizar un estudio comparativo de la diversidad genómica y patogénica de las cepas de *R. solanacearum* que afectan los cultivos de papa en Uruguay.
2) Identificar genes que contribuyen a la virulencia de *R. solanacearum*.
3) Identificar nuevos blancos genéticos para la detección de *R. solanacearum* por PCR.

El primer objetivo específico se desarrolla en el capítulo 2, dónde se presentan los resultados de caracterización de una colección de cepas uruguayas de *R. solanacearum*, aisladas a partir de diferentes zonas y zafras de producción de papa. El análisis comparativo de la agresividad de las cepas se realizó sobre diferentes

hospederos, incluyendo tomate, papa y accesiones de la especie silvestre *S. commersonii*. La diversidad genética de las cepas se evaluó mediante las técnicas moleculares BOX-PCR e hibridación genómica comparativa (CGH) sobre un chip de *microarrays* desarrollado en el Laboratorio de Interacciones Planta-Micoorganismo del INRA de Toulouse. Esta técnica permite realizar comparaciones genómicas a gran escala y detectar variaciones en el contenido genético incluso entre cepas muy relacionadas. La información resultante del estudio genómico comparativo se analizó con dos enfoques diferentes:

En el capítulo 3, se integraron los resultados del análisis por CGH y de la caracterización de la agresividad de las cepas, con el objetivo de identificar genes involucrados en la virulencia de *R. solanacearum*.

En el capítulo 4, esta información se utilizó para establecer una lista de genes específicos del tipo de cepas de *R. solancearum* presentes en Uruguay, los cuales se aplicaron al desarrollo de métodos moleculares de detección.

REFERENCIAS

Aldon D., Brito B., Boucher B, y Genin S. 2000. A bacterial sensor of plant cell contact controls the transcriptional induction of *Ralstonia solanacearum* pathogenicity genes. EMBO J. 19:2304-2314.

Alfano J.R. y Collmer A. 2004. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. Ann. Rev. Phytopathol. 42: 385-414.

Allen C., Huang Y.,, y Sequeira, L. 1991. Cloning of genes affecting polygalacturonase production in *Pseudomonas solanacearum.* Mol. Plant-Microbe Interact. 4:147-154.

Allen C., Gay J., y Simon-Buela, L. 1997. A regulatory locus, *pehSR,* controls polygalacturonase production and other virulence functions in *Ralstonia solanacearum.* Mol. Plant-Microbe Interact. 10:1054-1064.

Allen C., Kelman A., y French E.R. 2001. Brown rot of potatoes. Pages 11–13 in: Compendium of potato diseases, 2nd ed. W.R. Stevenson, R. Loria, G.D. Franc, y D.P. Weingartner, eds., APS Press, St. Paul, MN.

Álvarez B., López M.M., y Biosca E.G. 2008a. Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. Microbiology. 154:3590–3598.

Álvarez B., Vasse J., Le-Courtois V., Trigalet-Démery D., López M.M., y Trigalet A. 2008b. Comparative behavior of *Ralstonia solanacearum* biovar 2 in diverse plant species. Phytopathology. 98(1):59-68.

Angot A., Peeters N., Lechner E., Vailleau F., Baud C., Gentzbittel L., Sartorel E., Genschik P., Boucher C., y Genin S. 2006. *Ralstonia solanacearum* requires F-box-like domain-containing type III effectors to promote disease on several host plants. Proc. Natl. Acad. Sci. USA. 103:14620-14625.

Anguiz R.J. y Mendoza H.A. 1997. General and specific combining abilities for resistance to bacterial wilt (*Pseudomonas solanacearum* E.F. Smith) in PVX and PVY immune autotetraploid potatoes. Fitopatologia. 32: 71-80.

Anonymous. 2006. Commission Directive 2006/63/EC of 14 July 2006: amending Annexes II to VII to Council Directive 98/57/EC on the control of *Ralstonia solanacearum* (Smith) Yabuuchi et al. Off. J. Eur. Communities, L206, 36-106.

Arahal D.R., Llop P., Pérez Alonso M., y López M.M. 2004. In silico evaluation of molecular probes for detection and identification of *Ralstonia solanacearum* and *Clavibacter michiganensis* subsp. *sepedonicus*. System. Appl. Microbiol. 27:581-591.

Araud-Razou I., Vasse J., Montrozier H., Etchebar C., y Trigalet A. 1998. Detection and visualization of the major acidic exopolysaccharide of *Ralstonia solanacearum* and its role in tomato root infection and vascular colonization. Eur. J. Plant Pathol. 104:795-809.

Bamberg L.B., Martin M.W., y Schartner J.J. 1994. Elite selections of tuber-bearing *Solanum* especies germoplasm. Inter-regional Potato Introduction Station, NRSP-6, Sturgeon Bay, Wisconsin.

Bertolla F., Van Gijsegem F., Nesme X., Simonet P. 1997. Conditions for natural transformation of *Ralstonia solanacearum*. Appl. Environ. Microbiol. 63:4965–4968.

Bhatt G. y Denny T.P. 2004. *Ralstonia solanacearum* iron scavenging by the siderophore staphyloferrin B is controlled by PhcA, the global virulence regulator. J. Bacteriol. 186:7896-7904.

Boshou L. 2005. A broad review and perspective on breeding for resistance to bacterial wilt. Pages 225-238 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A. C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Boudazin G., Le Roux A.C., Josi K., Labarre P., y Jouan B. 1999. Design of division specific primers of *Ralstonia solanacearum* and application to the identification of European isolates. Eur. J.Plant Pathol.105:373–380.

Brown D., y Allen C. 2004. Ralstonia solanacearum genes induced during growth in tomato: an inside view of bacterial wilt. Mol. Microbiol. 53:1641-1660.

Brumbley S.M., Carney B.F., y Denny, T.P. 1993. Phenotype conversion in *Pseudomonas solanacearum* due to spontaneous inactivation of PhcA, a putative LysR transcriptional activator. J. Bacteriol. 175:5477-5487.

Buddenhagen I., Sequeira L., y Kelman A. 1962. Designation of races in *Pseudomonas solanacearum*. Phytopathology 52:726.

Canale, F. 1984. Situación de Pseudomonas solanacearum en la República Oriental del Uruguay. Pages 27-33 in: Seminario de Investigación y de Trabajo Sobre Avances en el Control de la Marchitez Bacteriana (Pseudomonas solanacearum) de la Papa en América Latina, Lima, Perú.

Canale, F., Peralta, A. 1983. Determinación de biotipos de Pseudomonas solanacearum en aislamientos procedentes de la zona sur de San José. Revista AIA 1(2):90-93.

Carney B.F., y Denny T.P. 1990. A cloned avirulence gene from *Pseudomonas solanacearum* determines incompatibility on *Nicotiana tabacum* at the host species level. J. Bacteriol. 172:4836-4843.

Carputo D., Aversano R., Barone A., Di Matteo A., Iorizzo M. Sigillo L., Zoina A., y Frusciante L. 2009. Resistance to *Ralstonia solanacearum* of sexual hybrids between *Solanum commersonii* and *S. tuberosum*. Am. J. Pot Res. 86:196–202

Caruso P., Gorris M.T., Cambra M., Palomo J.L., Collar J., y López M.M. 2002. Enrichment DASI-ELISA for sensitive detection of *Ralstonia solanacearum* in asymptomatic potato tubers using a specific monoclonal antibody. App. Environ. Microbiol.68: 3634–3638.

Caruso P., Bertolini E., Cambra M., y López M.M. 2003. A new and sensitive co-operational polymerase chain reaction (Co-PCR) for a rapid detection of *Ralstonia solanacearum* in water. J. Microbiol. Methods, 55:257-272.

Caruso P., Palomo J.L., Bertolini E., Álvarez B., López, M.M., y Biosca E.G. 2005. Seasonal variation of *Ralstonia solanacearum* biovar 2 populations in a Spanish river: Recovery of stressed cells at low temperatures. App. Environ. Microbiol. 71:140-148.

Castillo J.A., y Greenberg J.T. 2007. Evolutionary dynamics of *Ralstonia solanacearum*. Appl. Environ. Microbiol. 73: 1225–1238.

Ciampi, L., y Sequeira, L. 1980. Influence of temperature on virulence of race 3 strains of *Pseudomonas solanacearum* potatoes. Am. Pot. J. 57: 307-317.

Clough S.J., Lee K.-E., Schell, M.A., y Denny, T.P. 1997. A two-component system in *Ralstonia {Pseudomonas) solanacearum* modulates production of PhcAregulated virulence factors in response to 3-hydroxypalmitic acid methyl ester. J. Bacteriol. 179:3639-3648.

Colburn-Clifford J., y Allen. C. 2010. A *cbb3*-type cytochrome c oxidase contributes to *Ralstonia solanacearum* R3bv2 growth in microaerobic environments and to bacterial wilt disease development in tomato. Mol. Plant-Microbe Interact. 23:1042-1052.

Colburn-Clifford J., Scherf J.M., y Allen C. 2010. *Ralstonia solanacearum* Dps contributes to oxidative stress tolerance, colonization, and virulence on tomato plants. Appl. Environ. Microbiol. doi:10.1128/AEM.01742-10.

Cook D., Barlow E., y Sequeira L. 1989. Genetic diversity of *Pseudomonas solanacearum*: detection of restriction fragment length polymorphisms with DNA probes that specify virulence and the hypersensitive response. Mol. Plant- Microbe Interact. 2: 113-121.

Coutinho T.A. 2005. Introduction and prospectus of the survival of *R. solanacearum*. Pages 29-38 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A. C. Hayward, eds. Am Phytopathological Society, St. Paul, MN.

Cunnac S., Boucher C., y Genin S. 2004. Characterization of the cisacting regulatory element controlling HrpB-mediated activation of the type III secretion system and effector genes in *Ralstonia solanacearum*. J. Bacteriol. 186:2309-2318.

Delaspre F., Nieto-Penalver C.G., Saurel O., Kiefer P., Gras E., Milon A., Boucher C., Genin S., y Vorholt J.A. 2007. The *Ralstonia solanacearum* pathogenicity regulator HrpB induces 3-hydroxy-oxindole synthesis. Proc. Natl. Acad. Sci. USA. 104:15870-15875.

Denny T.P. 2005. A short history of the biochemical and genetic research on *Ralstonia solanacearum* pathogenesis. Pages 323-334 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A. C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Denny T.P. 2006. Plant pathogenic *Ralstonia* species. Pages 573-644. in: Plant-Associated Bacteria. S.S. Gnanamanickam ed. Springer Publishing, Dordrecht, The Netherlands.

Denny T.P., Makini F.W., y Brumbley S.M. 1988. Characterization of *Pseudomonas solanacearum* Tn5 mutants deficient in extracellular polysaccharide. Mol. Plant-Microbe Interact. 1:215-223.

Denny T.P., Carney B., y Schell M. 1990. Inactivation of multiple virulence genes reduces the ability of *Pseudomonas solanacearum* to cause wilt symptoms. Mol. Plant-Microbe Interact. 3:293-300.

Denny T.P. y Baek S.R 1991. Genetic evidence that extracellular polysaccharide is a virulence factor of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 4:198-206.

Deslandes L., Olivier J., Theuliéres F., Hirsch J., Feng D.X., Bittner-Eddy P., Beynon J., y Marco Y. 2002. Resistance to *Ralstonia solanacearum* in *Arabidopsis thaliana* is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. Plant Biol. 99:2404-2409.

DIEA-MGAP. Caracterización de la producción de papa en Uruguay. http://www.mgap.gub.uy/Diea/Encuestas/default.htm#Papera

Dorrell N., Hinchliffe S.J., y Wren B.W. 2005. Comparative phylogenomics of pathogenic bacteria by microarray analysis. Curr. Op. Microbiol. 8:620–626.

Ehrenreich A. 2006. DNA microarray technology for the microbiologist: an overview. Appl. Microbiol. Biotechnol. 73: 255-273.

Elphinstone J.G. 1996. Survival and possibilities for extinction of *Pseudomonas solanacearum* (Smith) Smith in cool climates. Pot. Res.39:403-410.

Elphinstone J.G. 2005. The current bacterial wilt situation: a global overview. Pages 9-28 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A. C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Elphinstone J.G., Hennessey J., Wilson J.K., y Stead D.E. 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. EPPO Bull. 26:663–78

Elphinstone J.G., Stanford H.M., y Stead D.E. 1998. Detection of *Ralstonia solanacearum* in potato tubers, *Solanum dulcamara*, and associated irrigation water. Pages 133–139 in: Bacterial Wilt Disease: Molecular and Ecological Aspects. P. Prior, C. Allen, y J. Elphinstone, eds. Springer-Verlag, Berlin.

Estrada Ramos N. 1995. La biodiversidad en el mejoramiento de la papa. Editorial Bogotá. Bolivia. 372 pp.

Fegan M., y Prior P. 2005. How complex is the '*Ralstonia solanacearum*' species complex? Pages 449- 461 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A. C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Fegan M., Holoway G., Hayward A.C. y J. Timmis. 1998. Development of a diagnostic test based on the polymerase chain reaction (PCR) to identify strains of *R. solanacearum* exhibiting the biovar 2 genotype. Pages 34-43 in: Bacterial Wilt Disease: Molecular and Ecological Aspects. P. Prior, C. Allen, y J. Elphinstone, eds. Springer-Verlag, Berlin.

Flavier A.B., Clough S.J., Schell M.A., y Denny T.P. 1997. Identification of 3-hydroxyplamitic methyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum.* Mol. Microbiol. 26:251-259.

Flores-Cruz Z., y Allen C. 2009. *Ralstonia solanacearum* encounters an oxidative environment during tomato infection. Mol. Plant-Microbe Interact. 22:773-782.

Floyd J. 2008. New Pest Response Guidelines: *Ralstonia solanacearum* race 3 biovar 2. USDA–APHIS–PPQ–Emergency y Domestic Programs, Riverdale, Maryland. http://www.aphis.usda.gov/import_export/plants/manuals/index.shtml

Fock I., Collonier C., Purwito A., Luisetti J., Souvannavong V., Vedel F., Servaes A., Ambroise A., Kodja H., Ducreux G., y Sihachakr D. 2000. Resistance to bacterial wilt in somatic hybrids between *Solanum tuberosum* and *Solanum phureja*. Plant Sci. 160:165-176.

French E.R., y De Lindo L. 1982. Resistance to *Pseudomonas solanacearum* in potato: specificity and temperature sensitivity. Phytopathology. 72:1408-1412.

French, E.R. 1994. Strategies for integrated control of bacterial wilt of potatoes. Pages 199-207 in: Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum.* A.C. Hayward y G.L. Hartman eds. CAB International, Wallingford, UK.

Gabriel D.W., Allen C., Schell M., Denny T.P., Greenberg J.T., Duan Y.P., Flores-Cruz Z., Huang Q., Clifford J.M., Presting G., González E.T., Reddy J., Elphinstone J., Swanson J., Yao J., Mulholland V., Liu L., Farmerie W., Patnaikuni M., Balogh B., Norman D., Alvarez A., Castillo J.A., Jones J., Saddler G., Walunas T., Zhukov A., y Mikhailova, N. 2006. Identification of open reading frames unique to a select agent: *Ralstonia solanacearum* race 3 biovar 2. Mol. Plant-Microbe Interact. 19:69-79.

Galván G., Franco Fraguas L., Quirici L., Santos C., Silvera E., Siri M.I., Villanueva P., Raudiviniche L., González M., Torres D., Castillo A., Dalla Rizza M., Vilaró F., Gepp V., Ferreira F., Pianzzola M.J. 2007. *Solanum commersonii*: una especie con gran potencial para el mejoramiento genético de papa por resistencia a *Ralstonia solanacearum*. Pages 87-101 in: Avances de Investigación en Recursos Genéticos del Cono Sur II. A. Clausen, F. Condón, y A. Berreta, eds. PROCISUR - IICA, Montevideo.

Garg R.P., Huang J., Yindeeyoungyeon W., Denny T.P., y Schell M.A. 2000. Multicomponent transcriptional regulation at the complex promoter of the exopolysaccharide I biosynthetic operon of *Ralstonia solanacearum*. J. Bacteriol. 182:6659-6666.

Genin S*.* 2010*.* Molecular traits controlling host range and adaptation to plants in *Ralstonia solanacearum.* New Phytologist.187:920–928*.*

Genin S., Gough C.L., Zischek C., y Boucher C.A. 1992. Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum.* Mol. Microbiol. 6:3065-3076.

Genin S., y Boucher C.A. 2002. *Ralstonia solanacearum*: secrets of a major pathogen unveiled by analysis of its genome. Mol. Plant Pathol.3:111–118.

Genin, S., y Boucher C.A. (2004). Lessons learned from the genome analysis of *Ralstonia solanacearum*. Ann. Rev. Phytopathol.42:107-134.

Genin S., Brito B., Denny T.P., y Boucher, C.A. 2005. Control of the *Ralstonia solanacearum* type III secretion system (Hrp) genes by the global virulence regulator PhcA. FEBS Lett. 579:2077-2081.

González M. 2010. Tesis de Maestría en Ciencias Agrarias. Facultad de Agronomía, Universidad de la República. Montevideo, Uruguay.

González E.T., y Allen C. 2003. Characterization of a *Ralstonia solanacearum* operon required for polygalacturonate degradation and uptake of galacturonic acid. Mol. Plant-Microbe Interact. 16:536-544.

Grey B.E., y Steck T.R. 2001. The viable but nonculturable state of *Ralstonia solanacearum* may be involved in long-term survival and plant infection. Appl. Environ. Microbiol. 67:3866-3872.

Griep R.A., Van Twisk C., Van Beckhoven J.R.C.M., Van der Wolf J.M., y Schots A. 1998. Development of specific recombinant monoclonal antibodies against the lipopolysaccharide of *Ralstonia solanacearum* race 3. Phytopathology 88:795-803

Guidot A., Prior P., Schoenfeld J., Carrere S., Genin S., y Boucher C.A. 2007. Genomic structure and phylogeny of the plant pathogen *Ralstonia solanacearum* inferred from gene distribution analysis. J. Bacteriol. 189:377-387.

Guidot A., Elbaz M., Carrere S., Siri M.I., Pianzzola M.J., Prior P. y Boucher C. 2009. Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathology 99:1105–1112.

Hakenbeck R., Balmelle N., Weber B., Gardes C., Keck W. y de Saizieu A. 2001. Mosaic genes and mosaic chromosomes: intra- and interspecies genomic variation of *Streptococcus pneumoniae*. Infect. Immun. 69: 2477-2486.

Hayward A.C. 1964. Characteristcs of *Pseudomonas solanacearum.* J Appl. Bacteriol. 27:265–77.

Hayward A.C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum.* Annu. Rev. Phytopathol. 29:65–87.

Hayward A.C. 1994a. Systematics and phylogeny of *Pseudomonas solanacearum* and related bacteria. Pages 123-135 in: Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A.C. Hayward y G.L. Hartman, eds. CAB International, Wallingford, UK.

Hayward A.C. 1994b. The hosts of *Pseudomonas solanacearum*. Pages 9-24 in: Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A.C. Hayward y G.L. Hartman, eds. CAB International, Wallingford, UK.

Hawkes J.G. 1991. The importance of genetic resources in plant breeding. Biol. Journal Linn. Soc. Lon. 43:3-10.

He Y.L., Sequeira L., y Kelman A. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Dis. 67:1357-1361.

Hershko A., y Ciechanover, A. 1998. The ubiquitin system. Annu. Rev. Biochem. 67:425-479.

Hirsch J., Deslandes L., Feng D., Balague C., Marco Y. 2002. Delayed symptom development in *ein2-1*, an *Arabidopsis* ethylene insensitive mutant, in response to bacterial wilt caused by *Ralstonia solanacearum*. Phytopathology 92:1142-1148.

Hotopp J.C., Grifantini R., Kumar N., Tzeng Y.L., Fouts D., Frigimelica E., Draghi M., Giuliani M.M., Rappuoli R., Stephens D.S., et al. 2006. Comparative genomics of *Neisseria meningitidis*: core genome, islands of horizontal transfer and pathogen-specific genes. Microbiology 152:3733-3749.

Huamán Z., Golmirzaie A., y Amoros W. 1997. The Potato. Pages 21-28 in: Biodiversity in trust. D. Fuccillo, L. Sears y P. Stapleton, eds. Cambridge University Press.

Huang J., Carney B.F., Denny T.P., Weissinger A.K., y Schell M.A. 1995. A complex network regulates expression of *eps* and other virulence genes of *Pseudomonas solanacearum.* J. Bacteriol. 177:1259-1267.

Huang Q., y Allen C. 1997. An exo-poly-alpha-D-galacturonosidase, PehB, is required for wild-type virulence of *Ralstonia solanacearum.* J. Bacteriol. 179:7369-7378.

Huang J., Wu J., Li C., Xiao C., y Wang G. 2009. Specific and sensitive detection of *Ralstonia solanacearum* in soil with quantitative, real-time PCR assays. J. Appl. Microbiol. 107:1729–1739.

Hughes T.R., Marton M.J., Jones A.R., et al., 2000. Functional discovery via a compendium of expresión profiles. Cell 102: 109-126.

Imazaki I., y Nakaho K. 2010. Pyruvate-amended modified SMSA medium: improved sensitivity for detection of *Ralstonia solanacearum*. J. Gen. Plant Pathol. 76(1): 52–61.

INASE. Estándares específicos para la producción de materiales de propagación de papa (Solanum tuberosum) de la clase certificada. Instituto Nacional de Semillas. http://www.inase.org.uy/especiesCultivares/EstandaresEspecificos.aspx

Janse J.D., Van der Beld H.E., Elphinstone J., Simpkins S., Tjou-Tam-Sin N.N.A., Van Vaerenbergh J. 2004. Introduction to Europe of *Ralstonia solanacearum* biovar 2, race 3 in *Pelargonium* zonale cuttings. J. Plant Pathol. 86:147–155.

Jaunet T.X., y Wang J.-F. 1999. Variation in genotype and aggressiveness diversity of *Ralstonia solanacearum* race 1 isolated from tomato in Taiwan. Phytopathology 9:320–327.

Kao C.C, Barlow E., y Sequeira L. 1992. Extracellular polysaccharide is required for wild-type virulence of *Pseudomonas solanacearum.* J. Bacteriol. 174:1068-1071.

Kang Y.W., Huang J.Z., Mao G.Z., He L.Y., Schell M.A. 1994. Dramatically reduced virulence of mutants of *Pseudomonas solanacearum* defective in export of extracellular proteins across the outer membrane. Mol. Plant-Microbe Interact. 7:370-377.

Kang Y., Liu H., Genin S., Schell M.A., y Denny T.P. 2002. *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. Mol. Microbiol. 46:427-437.

Keen N.T. 1990. Gene-for-gene complementarity in plant-pathogen interactions. Ann. Rev. Genet. 24:447-463.

Kelman A. 1954. The relationship of pathogenicity *of Pseudomonas solanacearum* to colony appearance in tetrazolium medium. Phytopathology. 44:693-695.

Laferriere L.T., Helgeson J.P. y Allen C. 1999. Fertile *S. tuberosum* + *S. commersonii* somatic hybrids as sources of resistance to bacterial wilt caused by *R. solanacearum*. Theor. Appl. Genet. 98:1272-1278.

Lambert C.D. 2002. Agricultural bioterrorism protection act of 2002: Possession, use, and transfer of biological; agents and toxins; interim and final rule. (7 CFR Part 331). Federal Register 67:76908-76938.

Lavie M., Shillington E., Equiluz C., Grimsley N., y Boucher C.A. 2002. PopP1, a new member of the YopJ/AvrRxv family of type III effector proteins, acts as a host-specificity factor and modulates aggressiveness of *Ralstonia solanacearum*. Mol. Plant-Microbe Interact. 15:1058-1068.

Li Y., Zhang Z., Jia Y., Shen Y., He H., Fang R., Chen X., Hao X. 2008. 3-Acetonyl-3-hydroxyoxindole: a new inducer of systemic acquired resistance in plants. Plant Biotechnol. J. 6:301-308.

Lin C.-H., Hsu S.-T., Tzeng K.-C., y J.-F. Wang. 2009. Detection of race 1 strains of *Ralstonia solanacearum* in field samples in Taiwan using a BIO-PCR method. Eur. J. Plant Pathol. 124:75–85.

Liu H., Kang Y., Genin S., Schell M.A., y Denny T.P. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiology 147:3215-3229.

Liu H., Zhang S., Schell M.A., y Denny T.P. 2005. Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol. Plant-Microbe Interact. 18:1296-1305.

Liu H. y Denny T. 2007: Proteomics analysis indicates that *Ralstonia solanacearum* has a distinctive type II secretion system. Phytopathology 97:S66-S67.

López M.M. y Biosca E.G. 2005. Potato bacterial wilt management: new prospects for an old problem. Pages 205-224 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A.C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Marco-Noales E., Bertolini E., Morente C. y López M.M. 2008. Integrated approach for detection of nonculturable cells of *Ralstonia solanacearum* in asymptomatic *Pelargonium* spp. cuttings. Phytopathology, 98(8):949-955.

Marenda M., Brito B., Callard D., Genin S., Barberis P., Boucher C.A., y Arlat M. 1998. PrhA controls a novel regulatory pathway required for the specific induction of *Ralstonia solanacearum hrp* genes in the presence of plant cells. Mol. Microbiol. 27:437-453.

Mattick J.S. 2002. Type IV pili and twitching motility. Annu. Rev. Microbiol.56: 289-314.

McGarvey J.A., Bell C.J., Denny T.P., y Schell M.A. 1998. Analysis of extracellular polysaccharide I in culture and *in planta* using immunological methods: new insights and implications. Pages 157-163 in: Bacterial wilt disease: molecular and ecological aspects. P. Prior, C. Allen, y J. Elphinstone eds., Springer-Verlag, Berlin.

McGarvey J.A., Denny T.P., y Schell M.A. 1999. Spatial-temporal and quantitative analysis of growth and EPS I production by *Ralstonia solanacearum* in resistant and susceptible tomato cultivars. Phytopathology 89:1233-1239.

Mc. Laughlin, R.J. y Sequeira, L. 1989. Phenotypic diversity in strains of *Pseudomonas solanacearum* isolated from a single potato field in Northeastern Florida. 73:960-964.

Mukaihara T., Tamura N., Murata Y., y Iwabuchi, M. 2004. Genetic screening of Hrp type III-related pathogenicity genes controlled by the HrpB transcriptional activator in *Ralstonia solanacearum*. Mol. Microbiol. 54:863–875.

Mukaihara T. y Tamura N. 2009. Identification of novel *Ralstonia solanacearum* type III effectors proteins through translocation analysis of hrpB-regulated gene products. Microbiology 155:2235–2244.

Occhialini A., Cunnac S., Reymond N., Genin S. y Boucher C.A. 2005. Genomewide analysis of gene expression in *Ralstonia solanacearum* reveals that the *hrpB* gene acts as a regulatory switch controlling multiple virulence pathways. Mol. Plant Microbe. Interact. 18:938–949

Orgambide G., Montrozier H., Servin P., Roussel J., Trigalet-Demery D., y Trigalet A. 1991. High heterogeneity of the exopolysaccharides of *Pseudomonas solanacearum* strain GMI1000 and the complete structure of the major polysaccharide. J Biol. Chem. 266:8312-8321.

Opina N., Tavner F., Hollway G., Wang J.-F., Li T.-H., Maghirang R., Fegan M., Hayward, A.C., Krishnapillai V., Hong W.-F., Holloway B.W., y Timmis J.N. 1997.A novel method for development of species and strain specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). Asia-Pac J. Mol. Biol. 5:19-30.

Osbourn A.E., Barber C.E., Daniels M.J. 1987. Identification of plant-induced genes of the bacterial pathogen Xanthomonas campestris pathovar campestris using a promoter-probe plasmid. EMBO J. 6:23-28.

Ozakman, M. y Schaad N.W. 2003. A real-time BIO-PCR assay for detection of *Ralstonia solanacearum* race 3, biovar 2, in asympotamtic potato tubers. Can. J. Plant Pathol. 25:232-239.

Pastrik K.H. y Maiss E. 2000. Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. J. Phytopathol. 148:619– 626.

Pastrik K.H., Elphinstone J.G. y R. Pukall. 2002. Sequence analysis and detection of *Ralstonia solanacearum* by multiplex PCR amplification of 16S-23S ribosomal intergenic spcer region with internal positive control. Eur. J. Plant Pathol. 108: 831–842.

Pegg G.F. 1985. Life in a black hole-the micro-environment of the vascular pathogen. Trans. British Mycol. Soc. 85:1-20.

Pegg K. y Moffett M. 1971. Host range of the ginger strain *of Pseudomonas solanacearum* in Queensland. Aust. J. Exp. Agric. Anim. Husb. 11:696-698.

Pelicic V. 2008. Type IV pili: e pluribus unum? Mol. Microbiol. 68(4):827–837.

Pianzzola M.J., Zarantonelli L., González G., Franco Fraguas L., y Vázquez A. 2005. Genetic, phytochemical and biochemical analyses as tools for biodiversity evaluation of wild *Solanum commersonii* accessions. Biochem. Syst. Ecol. 33:67-78.

Pizarro-Cerdá J. y Cossart P. 2006. Bacterial adhesion and entry into host cells. Cell 124:715–727.

Poueymiro M y Genin S. 2009. Secreted proteins from *Ralstonia solanacearum*: a hundred tricks to kill a plant. Curr. Opin. Microbiol. 12:44–52.

Poussier S., Vandewalle P., y Luisetti J. 1999. Genetic diversity of African and worldwide strains of *Ralstonia solanacearum* as determined by PCR-restriction fragment length polymorphism analysis of the *hrp* gene region. App. Environ. Microbiol. 65:2184-2194.

Poussier S. y Luisetti J. 2000. Specific detection of biovars of *R. solanacearu*m in plant tissue by nested PCR. Eur. J. Plant Pathol. 106:255-265.

Poussier S., Prior P., Luisetti J., Hayward A.C., y Fegan M. 2000. Partial sequencing of the *hrpB* and endoglucanase genes confirms and expands the known diversity within the *Ralstonia solanacearum* species complex. Syst. Appl. Microbiol. 23:479-486.

Poussier S., Cheron J-J, Couteau A., y Luisetti J. 2002. Evaluation of procedures for reliable PCR detection of *Ralstonia solanacearum* in common natural substrates. J. Microbiol. Meth. 51:349-359.

Pradhanang P.M., Elphinstone J.G., y Fox R.T.V. 2000a. Identification of crop and weed hosts of *Ralstonia solanacearum* biovar 2 in the hill of Nepal. Plant Pathol. 49:403–413.

Pradhanang P.M., Elphinstone J.G. y R.T.V. Fox. 2000b. Sensitive detection of *Ralstonia solanacearum* in soil: a comparison of different detection techniques. Plant Pathol. 49: 414-422.

Preston G.M., Studholme D.J., y Caldelari I. 2005. Profiling the secretomes of plant pathogenic Proteobacteria. FEMS Microbiol. Rev. 29:331-360.

Prior P., Steva H., y Cadet P. 1990. Aggressiveness of strains of *Pseudomonas solanacearum* from the French West Indies (Martinique and Guadeloupe) on tomato. Plant Disease. 74:962-965

Priou S., Gutarra L., y Alley P. 1999. Highly sensitive detection of *Ralstonia solanacearum* in latently infected potato tubers by postenrichment enzyme-linked immunosorbent assay on nitrocellulose membrane. EPPO Bull. 29:117-125

Priou S., Torres R., Villar A., Gutarra L., y De Mendiburu F. 2001. Optimization of sample size for the detection of latent infection by *Ralstonia solanacearum* in potato seed tubers in the highlands of Peru. Pot. Res. 44:349–58.

Priou S., Aley P., y Gutarra L. 2005. Assessment of resistance to bacterial wilt in CIP advanced potato clones. Pages 261-267 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A.C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Priou S., Gutarra L., Aley P. 2006. An improved enrichment broth for the sensitive detection of *Ralstonia solanacearum* (biovar 1 and 2A) in soil using DAS-ELISA. Plant Pathol. 55:36–45.

Priou S., Gutarra L., Aley P., De Mendiburu F., y Llique R. 2010. Detection of Ralstonia solanacearum (biovar 2A) in stems of symptomless plants before harvest of the potato crop using post-enrichment DAS-ELISA. Plant Pathol. 59: 59–67.

Remenant B., Coupat-Goutaland B., Guidot A., Cellier G., Wicker E., Allen C., Fegan M., Pruvost O., Elbaz M., Calteau A., Salvignol G., Mornico D., Mangenot S., Barbe V., Médigue C. y Prior P. 2010. Genomes of three tomato pathogens within the *Ralstonia solanacearum* species complex reveal significant evolutionary divergence. BMC Genomics, 11:379

Robertson A.E., Wechter W.P., Denny T.P., Fortnum B.A., y Kluepfel D.A. 2004. Relationship between avirulence gene (*avrA*) diversity in *Ralstonia solanacearum* and bacterial wilt incidence. Mol. Plant-Microbe Interact. 17: 1376-1384.

Saddler G.S. 2005. Management of bacterial wilt disease. Pages 121-132 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A.C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Saile E., Schell M.A., y Denny T.P. 1997. Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. Phytopathology 87:1264-1271.

Salama N., Guillemin K., McDaniel T.K., Sherlock G., Tompkins L. y Falkow S. 2000. A whole-genome microarray reveals genetic diversity among *Helicobacter pylori* strains. Proc. Natl. Acad. Sci. USA. 97: 14668-14673.

Salanoubat M., Genin S., Artiguenave F., Gouzy J., Mangenot S., Arlat M., Billault A., Brottier P., Camus J.C., Cattolico L., Chandler M., Choisne N., Claudel-Renard C., Cunnac S., Demange N., Gaspin C., Lavie M., Moisan A., Robert C., Saurin W., Schiex T., Siguier P., Thebault P., Whalen M., Wincker P., Levy M., Weissenbach J. y Boucher C.A. 2002. Genome sequenceof the plant pathogen *Ralstonia solanacearum*. Nature 415:497-502.

Sanchez Perez A., Mejia L., Fegan M., y Allen C. 2008. Diversity and distribution of *Ralstonia solanacearum* strains in Guatemala and rare occurrence of tomato fruit infection. Plant Pathol. 57:320-331.

Sarkar S.F., Gordon J.S., Martin G.B., y Guttman D.S. 2006. Comparative genomics of host-specific virulence in *Pseudomonas syringae*. Genetics 174: 1041–1056.

Schell M.A. 1993. Molecular biology of the LysR family of transcriptional regulators. Annu.Rev. Microbiol. 47:597-626.

Schell M.A. 1996. To be or not to be: How *Pseudomonas solanacearum* decides whether or not to express virulence genes. Eur. J. Plant Pathol. 102:459-469.

Schell M.A. 2000. Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory array. Ann. Rev. Phytopathol. 38:263-292.

Schonfeld J., Heuer H., Van Elsas J.D., y Smalla K. 2003. Specific and sensitive detection of *Ralstonia solanacearum* in soil on the basis of PCR amplification of *fliC* fragments. Appl. Environ. Microbiol. 69(12):7248–7256.

Seal S.E., Jackson L.A., y Daniels M.J. 1992. Isolation of a *Pseudomonas solanacearum*-specific DNA probe by subtraction hybridization and construction of species-specific oligonucleotide primers for sensitive detection by the polymerase chain reaction. Appl. Environ. Microbiol. 58:3751–3758.

Seal S.E., Jackson L.A., Young J.P.W., Daniels M.J. 1993. Differentiation of *P. solanacearum*, *P. syzygii*, *P. pickettii* and the blood disease bacterium by partial 16 S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. J. Gen. Microbiol, 139:1587–1594.

Seal S.E., Taghavi M., Fegan N., Hayward A.C. y Fegan M. 1999. Determination of *Ralstonia* (*Pseudomonas*) *solanacearum* rDNA subgroups by PCR tests. Plant Pathol. 48:115-120.

Siri M.I. 2005. Estudio del sistema *Solanum commersonii* - *Ralstonia solanacearum* enfocado a la búsqueda de factores de resistencia. Tesis de Maestría. Biblioteca de Facultad de Química, NI: 28346, Universidad de la República. Montevideo, Uruguay.

Siri M.I., Villanueva P., Pianzzola M.J., Franco Fraguas L., Galván G., Acosta M., Ferreira F. 2005. *In vitro* antimicrobial activity of different accessions of *Solanum commersonii* Dun from Uruguay. Pot. Res. 47:127-138.

Siri M.I., Galván G., Quirici L., Villanueva P., Ferreira F., Franco Fraguas L., Pianzzola M.J. 2009. Molecular marker diversity and bacterial wilt resistance in wild *Solanum commersonii* accessions from Uruguay. Euphytica 165(2):371-382.

Skerman V.B.D., McGowan V., y Sneath P.H.A. 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225-420.

Smith E.F. 1896. A bacterial disease of tomato, eggplant, and Irish potato (*Bacterium solanacearum* nov. sp.). USDA Div. Veg. Physiol. Pathol. Bull. 12:191.

Smith E.F. 1920. The Brown Rot of Solanacea. Pages 177-201 in: Bacterial Diseases of Plants. W.B. Saunders Company, Phildelphia.

Smith D.S. y De Boer S.H. 2009. Implementation of an artificial reaction control in a TaqMan method for PCR detection of *Ralstonia solanacearum* race 3 biovar 2. Eur. J. Plant Pathol. 124:405–412.

Sung J. M.-L, Lloyd D.H., y Lindsay J.A. 2008. *Staphylococcus aureus* host specificity: comparative genomics of human versus animal isolates by multi-strain microarray. Microbiology 154:1949–1959.

Swanson J.K., Yao J., Tans-Kersten J.K., y Allen C. 2005. Behavior of *Ralstonia solanacearum* race 3 biovar 2 during latent active infection of geranium. Phytopathology 95:136-143.

Tans-Kersten J.K., Guan Y., y Allen C. 1998. *Ralstonia solanacearum* pectinmethylesterase is required for growth on methylated pectin but not for bacterial wilt virulence. Appl. Environ. Microbiol. 64:4918-4923.

Tans-Kersten J.K., Huang H., y Allen C. 2001. *Ralstonia solanacearum* needs motility for invasive virulence on tomato. J. Bacteriol. 183:3597-605.

Tans-Kersten J.K., Brown D., y Allen C. 2004. Swimming motility, a virulence trait of *Ralstonia solanacearum*, is regulated by FlhDC and the plant host environment. Mol. Plant-Microbe Interact. 17:686-695.

Thurston H.D. 1963. Bacterial wilt of potatoes in Columbia. Am. Pot. J. 40:381-390.

USDA-United States Department of Agriculture. 2004. *Ralstonia solanacearum* race 3 biovar 2. Southern Geranium. New Pest Response Guidelines. USDA, APHIS, PPQ. Riverdale, MD.

Vailleau F., Sartorel E., Jardinaud M.F., Chardon F., Genin S., Huguet T., Gentzbittel L., y Petitprez. M. 2007. Characterization of the interaction between the bacterial wilt pathogen *Ralstonia solanacearum* and the model legume plant *Medicago truncatula*. Mol. Plant Microbe. Interact. 20:159–167.

Valls M., Genin S., Boucher C. 2006. Integrated regulation of the type III secretion system and other virulence determinants in *Ralstonia solanacearum*. PLoS Pathogens 2:798–807.

Van Elsas J.D., Kastelein P., Van Bekkum P., Van der Wolf J.M., De Vries P.M., Van Overbeek L.S. 2000. Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathology 90:1358–1366.

Vasse J. 1995. Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum*. Mol. Plant-Microbe. Interact. 8:241–251.

Vasse J., Genin S., Frey P., Boucher C.A., y Brito B. 2000. The *hrpB* and *hrpG* regulatory genes of *Ralstonia solanacearum* are required for different stages of the tomato root infection process. Mol. Plant-Microbe Interact. 13:259-267.

Vázquez A., González G., Ferreira F., Moyna P., y Kenne L. 1997. Glycoalkaloids of *Solanum commeronii.* Euphytica 95:195-201.

Verdier E. 2009. Situación de *Ralstonia solanacearum* en Uruguay. Pages 84-87 in: Aislamiento, detección e identificación de la bacteria *Ralstonia solanacearum.* Pianzzola M.J. y Galván G., eds. Montevideo, Uruguay.

Villa J., Tsuchiya K., Horita M., Natural M., Opina N., y Hyakumachi M. 2005. Phylogenetic relationships of *Ralstonia solanacearum* species complex strains from Asia and other continents based on 16S rDNA, *egl*, and *hrpB* gene sequences. J. Gen. Plant Pathol. 71:39-46.

Yabuuchi E., Kosako Y., Hano I., Hotta H., y Nishiuchi Y. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia pickettii* (Ralston, Palleroni, y Douderoff 1973) comb, nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb nov. Microbiol. Immun. 39:897-904.

Yabuuchi E., Kosako Y., Oyaizu H., Yano I., Hotta H., Hashimoto Y., Ezaki T., y M. Arakawa. 1992. Proposal *of Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immun. 36:1251-1275.

Yao J. y Allen C. 2006. Chemotaxis is required for virulence and competitive fitness of the bacterial wilt pathogen *Ralstonia solanacearum*. J. Bacteriol. 188:3697-3708.

Weller S.A., Elphinstone J.G., Smith N.C., Boonham N., y Stead D.E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative multiplex, real time, fluorogenic PCR (TaqMan) assay. Appl. Environ. Microbiol. 66: 2853-2858.

Williamson L., Nakaho K., Hudelson B., y Allen C. 2002. *Ralstonia solanacearum* race 3, biovar 2 strains isolated from geranium are pathogenic on potato. Plant Dis. 86:987-991.

CAPÍTULO 2

Análisis comparativo de la diversidad genómica y patogénica de las cepas de *Ralstonia solanacearum* responsables de la marchitez bacteriana de la papa en Uruguay

---

Parte del contenido de este capítulo fue enviado para su publicación en Plant Disease el 3 de Setiembre de 2010 con el título:

Comparative aggressiveness analysis of *Ralstonia solanacearum* strains (phylotype II, sequevar 1) causing bacterial wilt of potato in Uruguay.

Autores: Siri M.I., Sanabria A., Pianzzola M.J.

RESUMEN

La marchitez bacteriana causada por *Ralstonia solanacearum* es una enfermedad importante que afecta al cultivo de papa (*Solanum tuberosum*) en todo el mundo. Según los reportes locales, el patógeno se encuentra ampliamente distribuido en Uruguay, sin embargo, hasta el momento no se ha hecho un relevamiento representativo de las cepas presentes. En este trabajo se presenta la primera caracterización de una colección de cepas de *R. solanacearum* aisladas de diferentes zonas y zafras de producción papera en Uruguay. Los resultados revelaron que todas las cepas pertenecen al filotipo IIB, secuevar 1 (IIB-1). Se realizó un análisis comparativo de la agresividad sobre tomate y papa, revelando por primera vez, diferentes niveles de agresividad entre cepas de *R. solanacearum* pertenecientes al grupo IIB-1. Los ensayos de agresividad se realizaron también sobre varias accesiones de *Solanum commersonii*, una especie silvestre nativa de Uruguay, considerada una valiosa fuente de germoplasma para la introducción de resistencia en nuevas variedades de papa. No se encontró una interacción significativa entre *R. solanacearum* y *S. commersonii*, y las diferencias en la agresividad fueron consistentes con los grupos previamente identificados para tomate y papa. Por otra parte, se observaron diferencias significativas en la respuesta a *R. solanacearum* entre las accesiones evaluadas, lo que confirma la existencia de varios niveles de resistencia a la marchitez bacteriana en *S. commersonii*. La diversidad genética de esta colección de aislamientos se analizó mediante marcadores BOX-PCR e hibridación genómica comparativa (CGH) sobre un chip de *microarrays* que representa el genoma de *R. solanacearum.* Globalmente estos análisis revelaron una baja diversidad genética entre las cepas que actualmente están presentes en Uruguay y confirmaron su clasificación como IIB-1. La información generada mediante el análisis genómico comparativo por CGH permitió establecer el contenido genético de cada una de las cepas analizadas. Esta información resulta de utilidad para la identificación de genes relacionados con las diferencias de agresividad entre las cepas, así como para el desarrollo de herramientas epidemiológicas aplicables al control de este importante patógeno.

CHAPTER 2

Comparative genomic and pathogenic analyisis of *Ralstonia solanacearum* strains causing bacterial wilt of potato in Uruguay

---

Partial content of this chapter was submitted to Plant Disease on September 3, 2010, as:

Comparative aggressiveness analysis of *Ralstonia solanacearum* strains (phylotype II, sequevar 1) causing bacterial wilt of potato in Uruguay.

Authors: Siri M.I., Sanabria A., Pianzzola M.J.

ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* is a major disease affecting potato (*Solanum tuberosum*) production worldwide. Although local reports suggest that the disease is widespread in Uruguay, characterization of *R. solanacearum* strains prevalent in our country is scarcely documented. We present here the first evaluation of a collection of strains isolated from potato-growing areas in Uruguay. The survey revealed that all strains belong to the phylotype IIB, sequevar 1 (IIB-1), historically known as race 3, biovar 2. Pathogenicity tests on tomato and potato showed for the first time different levels of aggressiveness among *R. solanacearum* strains belonging to the IIB-1 group. Aggressiveness evaluation was also performed on several accessions of *Solanum commersonii*, a wild species native to Uruguay that is considered a valuable source of resistance for potato breeding. No significant interaction was found between strains and genotypes and differences in aggressiveness among *R. solanacearum* strains were consistent with previously identified groups based on tomato and potato experiments. Moreover, we observed different responses to *R. solanacearum* among the accessions tested, confirming the existence of different levels of bacterial wilt resistance in *S. commersonii*. Genetic diversity of Uruguayan strains was assessed by BOX-PCR and comparative genomic hybridization (CGH) using a microarray chip reflecting the pan-genome of *R. solanacearum*. These analysis revealed low genetic variability among Uruguayan strains and confirmed their classification as phylotype IIB, sequevar 1. The information generated through the genomic analysis by CGH allowed us to establish the genetic content of each tested strain. This information pave the way toward identifying candidate pathogenicity genes, and developing improved epidemiological tools to control this problematic pathogen.

INTRODUCTION

Bacterial wilt, caused by the bacterium *Ralstonia solanacearum* is a widespread disease affecting more than 200 plant species in tropical, subtropical and temperate regions of the world (Elphinstone, 2005; Hayward, 1994b). Species belonging to the family *Solanaceae* are particularly threatened, including economically important hosts such as tomato, potato, eggplant, pepper and tobacco (Hayward, 1991). *R. solanacearum* is a soil-borne pathogen that enters the plant through wounds in root tissues and progressively invades the stem vascular tissues, leading to partial or complete wilting and ultimately plant death. *R. solanacearum* is a complex species with exceptional diversity among strains regarding host range, geographical distribution, pathogenicity, epidemiological relationships, and physiological properties. Traditionally this complex species has been subdivided into five races on the basis of differences in host range (Buddenhagen et al., 1962; Pegg and Moffett, 1971; He et al., 1983) and six biovars on the basis of carbohydrate utilization (Hayward, 1964; 1994a). Although both classification schemes have been applied to describe strains worldwide, they have several disadvantages that limit their usefulness: they are time consuming, not able to discriminate at a sub-specific level, not predictive and do not correlate well with the phylogenetic origin of the strains (Denny, 2006).

Therefore, several molecular-based approaches have been developed to enhance the understanding of the genetic diversity of *R. solanacearum*. Recently, Fegan and Prior (2005) proposed a new hierarchical classification scheme based on phylogenetic analysis of the internal transcribed spacer (ITS) region and the endoglucanase gene, which proved to be more appropriate to reflect the strain diversity within the *R. solanacearum* species complex. Based on this system, four major groups, termed phylotpes, were distinguished which are related to the phylogenetic and geographic origin of strains. Phylotype I contain strains from Asia; phylotypes II, strains from the Americas; phylotypes III strains from Africa and surrounding islands; and phylotypes IV, strains isolated primarily from Indonesia, as well as two closely related species, *Ralstonia syzygii* and the banana blood disease bacterium (BDB). Each phylotype can

be further subdivided into a variable and additive number of sequevars, which are clusters of isolates with highly conserved DNA sequences. Using this hierarchical scheme, epidemiological and ecological groups of *R*. *solanacearum* strains can be distinguished, thereby allowing pathologists to better predict the biological properties of unknown strains and assist in the development of effective disease management strategies (Lewis et al., 2007).

In Uruguay, bacterial wilt caused by *R. solanacearum* is considered one of the most important diseases of potato, causing extensive damage and significant economic losses, especially when preventive control measures are not applied. The disease, commonly known as potato brown rot, also represents a limiting factor for potato seed production systems, which have a zero tolerance requirement for this pathogen. The first official report of the disease in Uruguay appeared in 1974 and the impact was devastating: 59% of the farmers were affected with losses varying between 5% and 90% of the total production. Since then, disease incidence has been variable and characterized by the occurrence of severe outbreaks, which are difficult to prevent and control. Moreover, the disease was reported throughout the country, avoiding the maintenance of pathogen-free areas for potato production. In temperate to cool regions in the world, potato brown rot is mainly produced by strains belonging to phylotype II, sequevar 1 (traditionally known as race 3, biovar 2) (Hayward 1994b). This group of strains is very homogeneous, possesses a narrow host range, and is highly virulent mainly for potatoes and tomatoes. To date, although local reports suggest that the pathogen is widespread in Uruguay, characterization of *R. solanacearum* strains prevalent in our country is scarcely documented. Based on a survey conducted during 1980s the presence of *R. solanacearum* strains belonging to biovar 2 was determined (E. Verdier, personal communication). However, this survey was discontinued and therefore the type of strains that currently affect potato crops in Uruguay is unknown.

As with other bacterial plant pathogens, the most effective control strategy for *R. solanacearum* remains the use of cultivars showing durable resistance. However, the performance of bacterial wilt resistance in potato seems relatively unstable across locations due to variation in environmental conditions and strain diversity (Boshou, 2005). This means that resistance breeding must be regionally targeted and breeders

must screen germplasm against locally prevalent strains of *R. solanacearum*. Wild relatives of cultivated potato provide a rich, unique and diverse source of genetic variation for potato breeding (Bradshaw et al., 2006). Extensive efforts have been made to transfer bacterial wilt resistance from various wild potato relatives. Disappointingly, sexual hybrids of potato with *S. chacoense*, *S. sparsipillum* and *S. multidissectum* accessions achieved only a moderate level of resistance, as well as some undesirable wild traits, such as high glycoalkaloid content (Boshou, 2005; French et al., 1998). A valuable genetic resource available for potato breeding in Uruguay is the wild species *Solanum commersonii* Dun. This tuber-bearing species native to Uruguay carries many desirable traits, such as tolerance to low temperatures and resistance to several pathogens (Carputo, et al., 2007, Tozzini et al., 1991). Some studies have reported the utilization of *S. commersonii* as a source of resistance to *R. solanacearum* (Cartputo et al., 2009; Laferriere, 1999). However, little is known about resistance across the whole spectrum of diversity in this wild species, nor about pathogenic fitness of different *R. solanacearum* strains. In a previous work, we evaluated a germplasm collection of *S. commersonii* accessions from different areas across the country (Siri et al., 2009). Interestingly, we found high genetic variation and different levels of bacterial wilt resistance. These findings highlight the potential of this species as a valuable genetic resource for potato breeding.

Knowledge on local pathogen populations is a key factor for successful breeding and integrated pest management program. The purpose of this study was to characterize *R. solanacearum* strains collected in Uruguay. Strains were identified with respect to biovar, phylotype and sequevar, and the genetic diversity was evaluated by rep-PCR and comparative genomic hybridization analyisis (CGH) using a microarray reflecting the pan-genome of *R. solanacearum.* In addition, we analyzed phenotypic variation of strains performing a comparative aggressiveness analysis on different hosts, including tomato, potato and several *S. commersonii* accessions differing in their resistance level. The information generated in this study will contribute to delineate control strategies for this important disease in Uruguay.

MATERIALS AND METHODS

Bacterial strains, isolation and growth conditions

Strains used in this study are listed in Table 2-1. *R. solanacearum* strains were isolated from potato tubers and soil samples collected from fields in the main areas of potato production in Uruguay during the period 2003-2009. Symptomatic tubers were washed thoroughly with water; surface-sterilized with 70% ethanol and a transverse section was made with a sterile scalpel. After pressing the tuber, a sterile loop was used to sample vascular exudates and streaked directly onto modified SMSA medium (mSMSA) (Elphinstone et al., 1996). Soil samples were sealed in a plastic bag and homogenized by hand shaking. Sub-samples of 2 g were incubated for 48 h in liquid mSMSA to enrich the number of *R. solanacearum* cells, and after this period isolation was performed in modified mSMSA agar. Plates were incubated for 3 to 5 days at 28 ºC and colonies with typical *R. solanacearum* phenotype (irregular shaped, fluidal and entirely white or with a pink centre) were subcultured onto tetrazolium chloride agar (TZC) (Kelman, 1954) and purified for further study.

Additionally, 11 untyped *R. solanacearum* strains wich had been conserved lyophilized were kindly provided by the governmental Plant Protection Service in Uruguay. These strains were isolated from potato tubers during a severe outbreak of potato brown rot in the 1980s and identified as *R. solanacearum* race 3, biovar 2. Reference strains IPO1609 and UW551 were kindly provided by C. Boucher (LIPM-INRA, Toulouse, France) and included as controls for the genetic and aggressiveness characterizations.

Identification of strains and long term storage

For each strain, a well isolated colony was selected and subjected to colony PCR using specific primers for *R. solanacearum* (Pastrik and Maiss, 2000; Seal et al., 1993). A loopful of suspected colonies was resuspended in 50 µl of sterile distilled water, boiled for 20 min, cooled on ice for 3 min and centrifuged at 6000 rpm for 1 min. PCR amplification was performed in a total volume of 25 µl containing 5 µl of boiled supernatant, 12.5 pmol of primers OLI1-Y2 or PS1- PS2, 1.5 mM $MgCl_2$, 200 of µM each dNTP, 1U of *Taq* DNA polymerase (Invitrogen) and the buffer supplied by the

manufacturer. Amplification was performed as follows: an initial denaturation step at 95ºC for 2 min, followed by 35 cycles of denaturation at 94ºC for 20 s, annealing at 68ºC for 20 s, extension at 72ºC for 30 s, and a final extension step at 72ºC for 5 min. PCR products (8 µl) were analyzed by electrophoresis through 1 % (w/v) agarose gels with 0.5 µg/ml ethidium bromide and photographed under UV light.

PCR positive strains were confirmed by pathogenicity tests on potato (*S. tuberosum* cv. Chieftain). Plantlets were micro-propagated from single-node pieces growing in vitro on Murashige and Skoog medium supplemented with 30 g of sucrose per liter and maintained at 22ºC and 16 h light/8 h dark. After three weeks, plantlets were transferred into plastic flats with 17 cm^3 cells containing commercial soil mix (TREF, Moerdijk, The Netherlands), and placed for acclimatization in a greenhouse at 22 to 25ºC under natural light for two weeks prior to inoculation. Four plants were inoculated with each *R. solanacearum* strain by direct stem inoculation, as previously described (Siri et al., 2009). Plants inoculated with saline solution were used as negative controls. Inoculated plants were held in a growth chamber at 28°C with 12 h photoperiod. When typical wilt symptoms were observed, the bacteria were re-isolated from the vascular tissue growing in mSMSA medium.

All *R. solanacearum* strains were long-term stored in casamino acid peptone glucose (CPG) broth (Hendrick and Sequeira, 1984) with 18 % glycerol at -70ºC. Individual isolates were also maintained as suspensions in sterile distilled water at room temperature.

Biovar determination

Uruguayan strains of *R. solanacearum* were classified to biovar using the physiological tests developed by Hayward (1964), which assay the ability of strains to oxidize a panel of sugars and sugar alcohols. Freshly cultured *R. solanacearum* cells were stabbed into a soft agar tube of Hayward's medium containing 1% (w/v) filter-sterilized lactose, maltose, cellobiose, manitol, sorbitol and dulcitol and incubated at 28ºC for 14 days. Each test was repeated two times and non inoculated tubes were used as negative controls. The colour change of the tubes was recorded daily. Acid production changed the colour of the culture medium from green to yellow.

DNA extraction

Bacterial growth from a well-separated single colony on TZC agar was used to inoculate 5 ml of liquid CPG. The culture was grown at 28ºC for 48 h with vigorous shaking. The entire suspension culture was then pelleted by centrifugation and total genomic DNA was extracted by standard procedures (Ausubel et al., 1994). Quality and quantity of DNA preparations were checked by gel electrophoresis and spectrophotometry, by measuring the absorbance at 260 and 280 nm. DNA samples were stored at -20ºC.

Phylotype identification

Phylotype affiliation of Uruguayan strains was determined by multiplex PCR using a set of phylotype-specific primers (Nmult:21:1F, Nmult:21:2F, Nmult:22:InF, Nmult:23:AF and Nmult21:RR) and species-specific primers (759, 760) (Fegan and Prior, 2005). Amplification was carried out in a total volume of 25 µl containing 50 ng of genomic DNA, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 6 pmol of each phylotype-specific primer, 4 pmol of species-specific primers, 2 U of *Taq* DNA polymerase (Invitrogen) and the buffer supplied by the manufacturer. Amplifications were performed with an initial denaturation step at 96ºC for 5 min, followed by 30 cycles of denaturation at 94ºC for 15 s, annealing at 59ºC for 30 s, extension at 72ºC for 30 s, and a final extension step at 72ºC for 10 min. PCR products (12 µl) were analyzed by electrophoresis through 2 % (w/v) agarose gels with 0.5 µg/ml ethidium bromide and photographed under UV light. Genomic DNA from strains GMI1000 (phylotype I), IPO1609 (phylotype II), CMR15 (phylotype III) and Psi07 (phylotype IV) provided by C. Boucher (LIPM-INRA, Toulouse, France) were used as positive amplification controls.

Sequevar identification

PCR amplification of a 750-bp fragment of the endoglucanase (*egl*) gene was performed using the primer pair Endo-F and Endo-R (Fegan et al., 1998). Amplifications were carried out in a total volume of 25 µl containing 50 ng of genomic DNA, 10 pmol of each primer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 U of *Taq* DNA polymerase (Invitrogen) and the buffer supplied by the manufacturer. Amplifications were

performed with an initial denaturation step at 96ºC for 9 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 70ºC for 1 min and extension at 72ºC for 2 min, and a final extension step at 72ºC for 10 min. PCR products were purified and sequenced by Macrogen services (Kumchun-ku, Seoul, Korea) using Endo-F and Endo-R primers. Sequences were edited and assembled with Vector NTI Advance 11.0 sequence analysis software package (Invitrogen) and deposited in the GenBank database under accession numbers HQ198559 to HQ198584. Phylogenetic analysis was performed with MEGA 3.0 (Kumar et al., 2004) by using neighbour-joining and the algorithm of Jukes and Cantor (Jukes and Cantor, 1969) with 1,000 bootstraps resamplings. The following 28 reference sequences were retrieved from GenBank database and included in the phylogenetic analysis aiming to cover the genetic diversity of *R. solanacearum* species complex: R292 (AF295255), NCPPB3190 (AF295253), GMI1000 (DQ657595), U154 (AY464996), MOD5 (AY464992), UW21 (DQ011546), UW469 (AF295269), UW477 (DQ657604), CFBP2958 (AF295266), CFBP2957 (EF371807), UW167 (DQ011545), UW276 (DQ657610), UW344 (DQ657620), UW551 (DQ657596), CFBP3858 (AF295259), JT516 (EF647737), CFBP3059 (DQ657647), J25 (AF295279), JT525 (AF295272), JT528 (AF295273), NCPPB283 (AF295275), NCPPB332 (DQ657649), NCPPB505 (AF295277), NCPPB1018 (AF295271), ICMP9915 (DQ011555), MAFF301558 (AY465002), R28 (DQ011552), R230 (AF295280) and R233 (DQ011542).

Evaluation of aggressiveness

Aggressiveness of a subset of *R. solanacearum* strains was determined on tomato (*Solanun lycopersicum* cv. Loica), potato (*S. tuberosum* cv. Chieftain) and several accessions of the wild potato relative, *S. commersonii*. In order to prepare the inocula, bacterial strains were grown overnight in liquid CPG at 28ºC while shaking at 200 r.p.m. Cells were pelleted by centrifugation, suspended in 0.9 % saline solution and spectrophotometrically adjusted to 10^8 cfu/ml (optical density at 600 nm of 0.1). Inoculum concentration was confirmed by dilution plating on TZC agar. Aggressiveness evaluation of 11 and 16 Uruguayan *R. solanacearum* strains was performed on tomato and potato respectively. Reference strains IPO1609 and UW551 were included in the

analysis as controls. Tomato seedlings were grown from seed in plastic flats with 17 cm^3 cells containing commercial soil mix (TREF, Moerdijk, The Netherlands). Plants were grown in a greenhouse at 22 to 25ºC under natural light for three weeks prior to inoculation. Potato plants were grown and propagated, as described below. Tomato and potato plants with 6 to 8 expanded leaves (approximately 10 cm high) were tested in separate experiments by soil inoculation (Montanelli et al., 1995). Prior to inoculation, roots were slightly damaged by making a hole with a 1000 µl disposable pipette tip next to each plant, and then one ml of the bacterial suspension was poured inside the hole (5 x 10^6 cfu/g soil). A set of 12 tomato plants and potato plants were inoculated with each strain and placed in separated trays in order to prevent cross contamination. Three replicate trays were inoculated with each strain in a completely randomized design. Plants inoculated with saline solution were used as negative controls in all experiments. After inoculation, plants were incubated in a growth chamber under 28ºC with 12h photoperiod. Disease development was recorded at regular time intervals using an ordinal scale ranging from 0 (no wilting symptoms) to 4 (all leaves wilted) up to 21 days after inoculation (Winstead and Kelman, 1952). Strain aggressiveness was estimated by the area under disease progression curve (AUDPC) based on average wilt scoring for each combination of strain and host (Sivleira et al., 1998). AUDPC data was subjected to analysis of variance (ANOVA) and means were compared using Tukey's multiple comparison procedure using INFOSTAT software (Di Rienzo et al., 2009). Scheffe's contrasts were calculated to compare means of groups of strains with different origin (Montgomery, 2005).

Thirteen *R. solanacearum* strains were tested on several *S. commersonii* accessions obtained from a germplasm collection maintained at the National Institute for Agricultural Research (INIA, Las Brujas, Uruguay). Three accessions that carried different levels of genetic resistance to *R. solanacearum* were selected based on a previous screening (Siri et al., 2009). Potato plants cv. 'Chieftain' were included as susceptible control. *S. commersonii* accessions were propagated by *in vitro* culture as described above with the exception that four to five weeks were required for rooting prior to transplanting. Plants with 6 to 8 expanded leaves were infected by soil inoculation. Preparation of the inocula, inoculation procedure, and incubation

conditions were the same as described above. Ten plants of each accession were inoculated with each strain in a randomized complete blocks design with two blocks. Plants inoculated with saline solution were used as negative controls. Disease development was recorded at regular time intervals for 21 days and AUDPC was calculated for each combination of strain and host based on average wilting scoring (Silveira et al., 1998). ANOVA was used to determine the effects of strain, host, and strain-host interactions on the AUDPC data. Means were compared using Tukey's multiple comparison test using INFOSTAT software (Di Rienzo et al., 2009).

BOX-PCR

Genetic diversity of Uruguayan *R. solanacearum* strains was determined by rep-PCR employing the BOXAIR primer (5'-CTACGGCAAGGCGACGCTGACG-3') (Louws et al., 1994). Reference potato strains UW551 and IPO1609 were also included in the analysis. Strain GMI1000 (phylotype I) was used as outgroup for the genetic analysis. PCR amplifications were performed in 25 µl reaction volumes containing 50 ng of genomic DNA, 50 pmol of primer BOXA1R, 6.8 mM $MgCl_2$, 200 of µM each dNTP, 2 U of *Taq* DNA polymerase (Invitrogen) and the buffer supplied by the manufacturer. Amplifications were performed with an initial denaturation step at 95ºC for 7 min, followed by 30 cycles of dentaturation at 94ºC for 1 min, annealing at 53ºC for 1 min, extension at 65ºC for 8 min, and a final extension step at 65ºC for 16 min. To confirm reproducibility of the results, amplifications were repeated twice for each bacterial strain. PCR products (12 µl) were analyzed by electrophoresis through 2 % (w/v) agarose gels with 0.5 µg/ml ethidium bromide and photographed under UV light. Gel images were analyzed with software Gel Compar 4.2 (Kortrijk, Belgium). The difference between the patterns generated was established by the presence (1) or absence (0) of an amplification product. The percentage of reproducibility was determined by dividing the number of reproducible bands by the total number of bands observed. Non-reproducible bands were not used for the analysis. Similarity matrix was constructed from the binary data with Dice's coefficients and dendogram was generated with UPGMA (unweighted pair-group method using arithmetic average)

clustering algorithm. The goodness of fit of the cluster analysis to the associated similarity matrix was computed by cophenetic correlation analysis.

Comparative genomic hybridization analysis

Microarray description:

The DNA microarray used in this study was generated by C. Boucher and collaborators (INRA-CNRS, Toulouse, France). This spotted microarray consists of 6515 65-mer and 70-mer oligonucleotides representative of the genes identified from the genomes of three *R. solanacearum* strains: GMI1000 (phylotype I, sequevar 18); IPO1609 (phylotype IIB, sequevar 1); and Molk2 (phylotype IIB, sequevar 3). The original chip contained 5074 oligonucleotides representative of the 5120 predicted genes from the first sequenced *R. solanacearum* strain GMI1000 (Occhialini et al., 2005). This chip was then completed with 1150 oligunucleotides that were identified from the complete genome sequence of two new sequenced *R. solanacearum* strains (IPO1609 and Molk2) (Guidot et al., 2009). These additional oligonucleotides represents genes absent in the genome sequence of strain GMI1000 or with no homolog in this strain (<80% amino acid identity or identity covering <80% of the total length of the two proteins). Each gene was represented by a single oligonucleotide except for 115 type three secretion system effectors genes, which were represented by 2 to 6 oligonucleotides to distinguish allelic forms of a given gene. A limited number of oligonucleotides representative of particular intergenic regions were also added to the microarray. In addition, the chip also includes as negative controls 10 oligonucleotides corresponding to five *Corynebacterium glutamicum* genes and a set of “blank” controls in which buffer without oligonucleotide was spotted. Each oligonucleotide was spotted twice on the chip.

DNA labeling and microarray hybridization:

Labeling and microarray hybridization were performed as previously described by Guidot et al. (2007), with minor modifications. Each experiment consisted of a competitive hybridization between genomic DNA from a tested strain and a standard control DNA labeled with either Cy3-dCTP or Cy5-dCTP fluorescent dyes (Amersham,

Biosciences). Standard control DNA used for all genome hybridization experiments consisted of an equimolar combination of the genomic DNA from the 3 sequenced strains GMI1000, IPO1609, and Molk2. Labeling of genomic DNA was done by using the BioPrime DNA labeling system kit (Invitrogen) according to the manufacturer's recommendations. For a 50-µl reaction mixture, 2 µg of genomic DNA in 23 µl of sterile water was heated at 95°C for 10 min, combined with 20 µl of 2.5× random primers solution, heated again at 95°C for 5 min, and chilled on ice. Remaining components were added to the following final concentrations: 0.12 mM dATP, dGTP, and dTTP; 0.06 mM dCTP; 0.02 mM Cy3- or Cy5-dCTP (Amersham, Biosciences); 1 mM Tris-HCl (pH 8.0); 0.1 mM EDTA; and 40 units of Klenow fragment (Invitrogen). The solution was incubated at 37°C for 2 h before the reaction was stopped by adding EDTA (pH 8.0) to a final concentration of 45 mM. The fluorescence-labeled probes were purified using the CyScribe GFX purification kit (Amersham Biosciences) and dissolved in 60 µl of elution buffer. Hybridizations were carried out using a Lucidea automated slide processor (Amersham Pharmacia Biotech). Each experiment was run as a competitive hybridization by using Cy3-labeled DNA from each tested strain and Cy5-labeled control DNA. No dye swapping was performed, since preliminary experiments had demonstrated that this had no significant impact on the final results (Guidot et al., 2007). Microarrays were prehybridized for 1 h at 42°C in Dig Easy buffer (Roche), containing 385 µg/ml of salmon sperm DNA. Hybridization was done for 15 h under the same conditions after 1 µg each of Cy3- and Cy5-labeled DNA were added. Following hybridization, microarrays were washed in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate for 5 min at 60°C, and then in 0.1× SSC for 5 min at room temperature, dried at 37°C for 5 min, washed by immersion in isopropanol and finally dried at 37°C for 5 min.

Array scanning and analysis:

Hybridized microarrays were scanned using a GenePix 4000A dual-channel confocal laser scanner (Axon Instruments) at 532 nm (Cy3) and 645 nm (Cy5) excitation wavelengths with a resolution of 10 nm per pixel. Quantification of the signals from individual arrays and data analysis were performed with ImaGene and Genesight

software (BioDiscovery, Inc.). Spots were excluded from further analysis if they contained an anomalous spot morphology, impurities, high local background fluorescence, or weak intensity compared to the signal observed for hybridization to the negative controls. For each spot, the ratio of the hybridization signal of the tested strain to that of the reference control strains was calculated and $\log_2$ transformed, and the values were normalized as was previously optimized by Guidot et al. (2007). The presence or absence of a gene in each tested strain was determined by comparing the average $\log_2$ ratio value thus calculated with the cut-off value of -1. In other words, a gene was consider to be absent (or highly divergent) if the hybridization signal of the tested strain was at least 2 times weaker than the hybridization signal of the reference strains. The status for all genes was converted into trinary scores (present = 1; absent = 0; missing data = 2). The final data set for each strain consisting of the assigned trinary scores for all genes, were subjected to hierarchical clustering using Genesight 3.5.2 software (BioDiscovery, Inc.). The Ward technique was used for cluster linkage and the Euclidian method for distance metric. To examine the relation of Uruguayan *R. solanacearum* strains to the whole diversity available within the species complex, microarray CGH data for additional *R. solanacearum* strains belonging to all 4 phylotypes was kindly provided by C. Boucher (LIPM-INRA, Toulouse, France) and included in the analysis. CGH experiments for these strains were performed by A. Guidot and M. Elbaz (LIPM-INRA, Toulouse, France) following the same protocol described above.

RESULTS

Strain isolation, identification and characterization

Twenty-eight bacterial strains were collected from fields located in the main potato production regions in Uruguay during the period 2003-2009 (Table 2-1, Figure 2-1). Most of these isolates were obtained from potato tubers showing typical brown rot symptoms (creamy exudates from vascular rings and eyes of tubers). Two strains were isolated from soil samples collected in 2004 and 2007 from potato fields. Additionally, eleven *R. solanacearum* strains were obtained from the collection of the Plant Protection Service (DGSA) in Montevideo, Uruguay. These strains were isolated from potato tubers from a severe outbreak of potato brown rot in the 1980s, and showed an atypical phenotype in culture, as they grow as small, round dark red colonies on TZC medium (Figure 2-1).

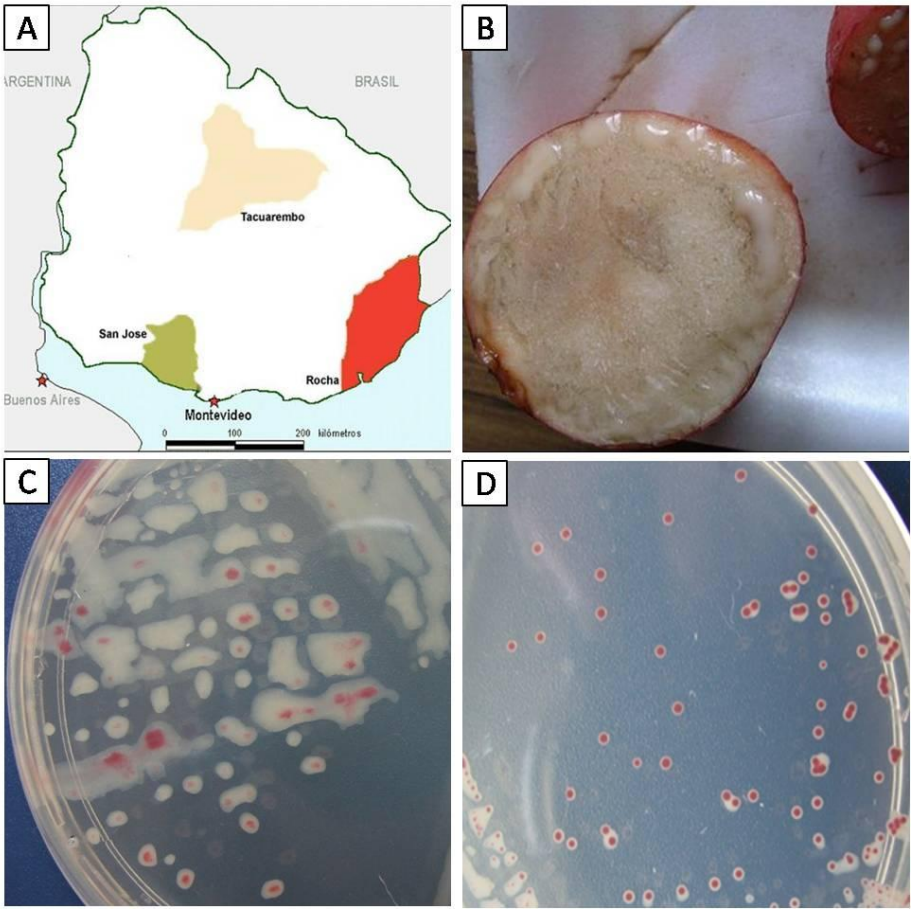


Figure 2-1. Isolation of Uruguayan *Ralstonia solanacearum* strains and phenotype in culture. A. Map showing the main areas of potato production in Uruguay. South: San

José (green); East: Rocha (red); North: Tacuarembó (pink). B. Cross-section of a potato tuber infected with *R. solanacearum*, showing partial grey-brown discoloration of vascular tissues, and a typical white creamy exudates or ooze along the vascular ring. C. Typical colony morphology of *R. solanacearum* in tetrazolium chloride agar (TZC) after incubation 72 h at 28ºC: irregular shaped colonies, fluidal and entirely white or with a pink centre (Kelman, 1954). D. Atypical phenotype in TZC observed for archival strains isolated in the 1980s growing as small, round dark red colonies.

Table 2-1. *Ralstonia solanacearum* strains used in this study

	Origin				Phylotype-Sequevar
Strain	**Year**	**Location[a]**	**Sample**	**Biovar**	
Uruguayan strains					
UY031	2003	San José	Potato tuber	2	IIB-1
UY032	2003	San José	Potato tuber	2	IIB-1
UY033	2003	Tacuarembó	Potato tuber	2	IIB-1
UY034	2003	Tacuarembó	Potato tuber	2	IIB-1
UY035	2003	Tacuarembó	Potato tuber	2	IIB-1
UY036	2003	Tacuarembó	Potato tuber	2	IIB-1
UY041	2004	Rocha	Potato tuber	2	IIB-1
UY042	2004	Rocha	Potato tuber	2	IIB-1
UY043	2004	Tacuarembó	Soil	2	IIB-1
UY071	2007	Rocha	Soil	2	II-ND
UY072	2007	Tacuarembó	Potato tuber	2	IIB-1
UY081	2008	San José	Potato tuber	2	IIB-1
UY082	2008	San José	Potato tuber	2	IIB-1
UY083	2008	San José	Potato tuber	2	IIB-1
UY084	2008	San José	Potato tuber	2	IIB-1
UY085	2008	San José	Potato tuber	2	IIB-1
UY086	2008	Rocha	Potato tuber	2	IIB-1
UY087	2008	Rocha	Potato tuber	2	IIB-1
UY088	2008	Rocha	Potato tuber	2	IIB-1
UY089	2008	Rocha	Potato tuber	2	IIB-1
UY0810	2008	Rocha	Potato tuber	2	IIB-1
UY091	2009	San José	Potato tuber	2	IIB-1
UY092	2009	San José	Potato tuber	2	II-ND
UY093	2009	San José	Potato tuber	2	II-ND
UY094	2009	San José	Potato tuber	2	II-ND
UY095	2009	Rocha	Potato tuber	2	II-ND
UY096	2009	Rocha	Potato tuber	2	II-ND
UY097	2009	Rocha	Potato tuber	2	II-ND

Table 2-1. Continue

Strain	Origin			Biovar	Phylotype-Sequevar
	Year	Location[a]	Sample		
DGSA114[b]	1980-1984	San José	Potato tuber	2	IIB-1
DGSA115[b]	1980-1984	San José	Potato tuber	2	IIB-1
DGSA116[b]	1980-1984	San José	Potato tuber	2	IIB-1
DGSA117[b]	1980-1984	San José	Potato tuber	2	IIB-1
DGSA119[b]	1980-1984	San José	Potato tuber	2	IIB-1
DGSA120[b]	1980-1984	San José	Potato tuber	2	IIB-1
DGSA127[b]	1980-1984	San José	Potato tuber	2	IIB-1
DGSA130[b]	1980-1984	San José	Potato tuber	2	IIB-1
DGSA132[b]	1980-1984	Tacuarembó	Potato tuber	2	IIB-1
DGSA133[b]	1980-1984	Tacuarembó	Potato tuber	2	IIB-1
DGSA135[b]	1980-1984	San José	Potato tuber	2	IIB-1
Reference strains [c]					
IPO1609	1995	The Netherlands	Potato tuber	2	IIB-1
UW551	2003	Kenya	Geranium	2	IIB-1

ND: not determined

[a] Strains were collected from all three potato-growing areas in Uruguay: San José (South), Tacuarembó (North) and Rocha (East). Strains belonged to different fields with the exception of strains UY031 and UY032 that were isolated from different tubers collected in the same field. [b] Strains isolated during the 1980s maintained at the collection of the Plant Protection Service in Uruguay. [c] Reference strains IPO1609 and UW551 were kindly provided by C. Boucher (LIPM- INRA, Toulouse, France).

All strains were confirmed as *R. solanacearum* by colony PCR, yielding fragments of the expected sizes following amplification with primers OLI1-Y2 and PS1-PS2 respectively (Pastrik and Maiss, 2000; Seal et al., 1993) (Figure 2-2). Furthermore, all strains isolated in this work produced typical symptoms of wilting on potato plants 4 to 5 days after direct stem inoculation (Figure 2-3). The strains were re-isolated from the stem flow of potato wilting plants and showed typical colony phenotype on TZC medium. In contrast, the remaining 11 strains isolated in the 1980s did not cause wilt symptoms on potato and no suspect bacteria were re-isolated from asymptomatic plants 14 days after inoculation. Based on these results we assumed that archival *R. solanacearum* strains had lost their virulence due to long-term storage so we decided to exclude them for the comparative aggressiveness analysis. Nevertheless, since these isolates were confirmed as *R. solanacearum* by PCR amplification they were included in the

genetic analysis for comparison with strains currently affecting potato crops in Uruguay.

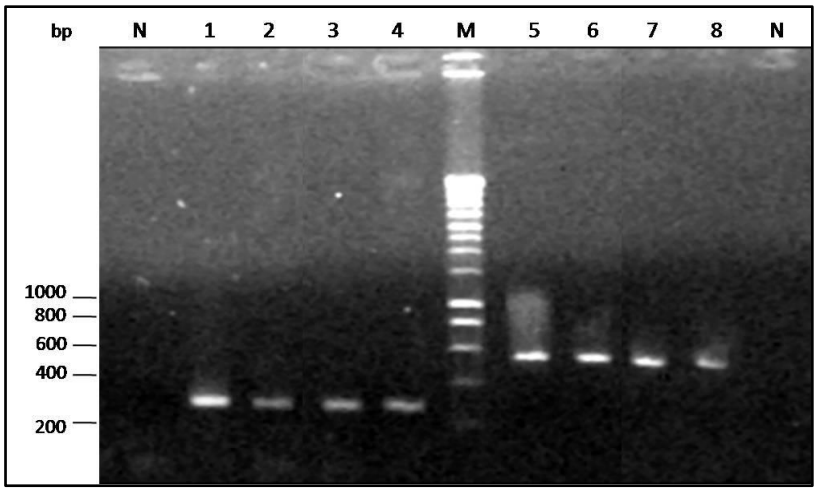


Figure 2-2. Representative results for the identification of Uruguayan *Ralstonia solanacearum* strains by PCR. Lanes 1-4: amplification of strains UY031, UY032, UY033 and UY034 with primers OLI1-Y2 yielding an expected 288-bp fragment (Seal et al., 1993). Lanes 2-8: amplification of strains UY031, UY032, UY033 and UY034 with primers PS1-PS2 yielding an expected 553-bp fragment (Pastrik and Maiss, 2000). N: negative control of PCR (without DNA). M: molecular weight marker (Samart Ladder Eurogentec, 10-kb).

Figure 2-3. Typical wilt symptoms observed in potato plants (cv. Chieftain) 4-5 days after stem inoculation with Uruguayan *Ralstonia solanacearum* strains.

All Uruguayan strains, typically metabolized disaccharides (lactose, maltose, cellobiose) but not hexose alcohols (manitol, sorbitol, dulcitol), and therefore were classified as biovar 2 (Table 2-1). In addition, all strains were characterized on the basis of the hierarchical classification system proposed by Fegan and Prior (2005). Phylotype-specific multiplex PCR resulted in the amplification of the expected 282-bp species-complex-specific fragment and an additional 372-bp amplicon (Figure 2-4). These results indicate that all strains in our Uruguayan's collection belong to phylotype II. The endoglucanase (*egl*) gene sequences were determined for 32 strains. Partial DNA sequences were aligned and compared with reference strains in order to locate Uruguayan strains within the known phylogenetic structure (Figure 2-5). Based on sequence analysis, all Uruguayan strains had identical *egl* sequences and were assigned to phylotype IIB - sequevar 1 group (IIB-1), resembling most other strains causing potato brown rot in temperate regions worldwide. This phylogenetic characterization is entirely consistent with the previous assignation as biovar 2.

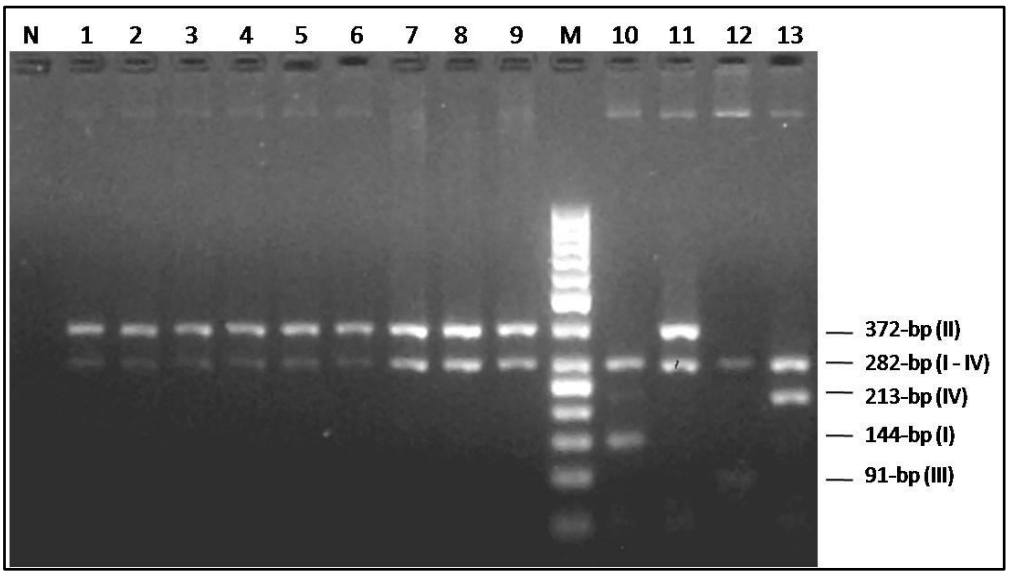


Figure 2-4. Representative results for the phylotype specific multiplex PCR reaction of Uruguayan *Ralstonia solanacearum* strains. Lanes 1-9: amplification of strains UY031, UY032, UY033, UY034, UY035, UY036, UY041, UY042 and UY043, lanes 10-13: positive amplification controls for phylotype I (strain GMI1000), II (strain IPO1609), III (strain CMR15) and IV (strains Psi07). All *R. solanacearum* strains amplified a 282-bp product and a phylotype specific product according to Fegan and Prior (2005). N: negative control of PCR (without DNA). M: molecular weight marker (Amersham Biosciences).

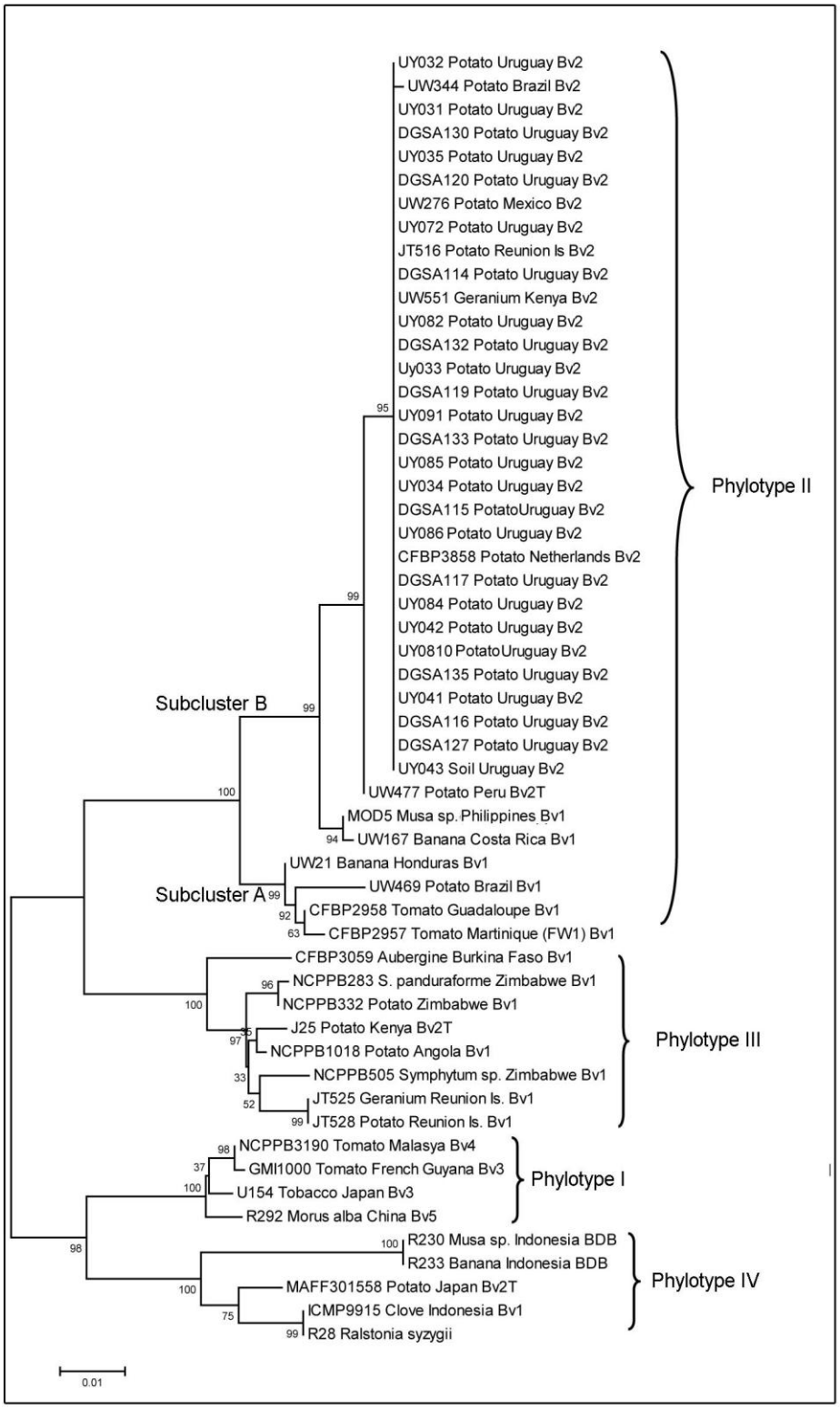


Figure 2-5. Phylogenetic neighbour-joining tree based on the partial (690-bp) endoglucanase (*egl*) gene sequences of *Ralstonia solanacearum* strains from Uruguay and reference strains from the species complex. The scale bar represents one nucleotide substitution per 100 nucleotides.

Comparative aggressiveness analysis

Aggressiveness analysis was performed for a subset of strains covering different potato regions in Uruguay and years of isolation. Strain aggressiveness was tested first on tomato and potato by a soil inoculation procedure. An overview of the experimental set up is showed in Figure 2-6.

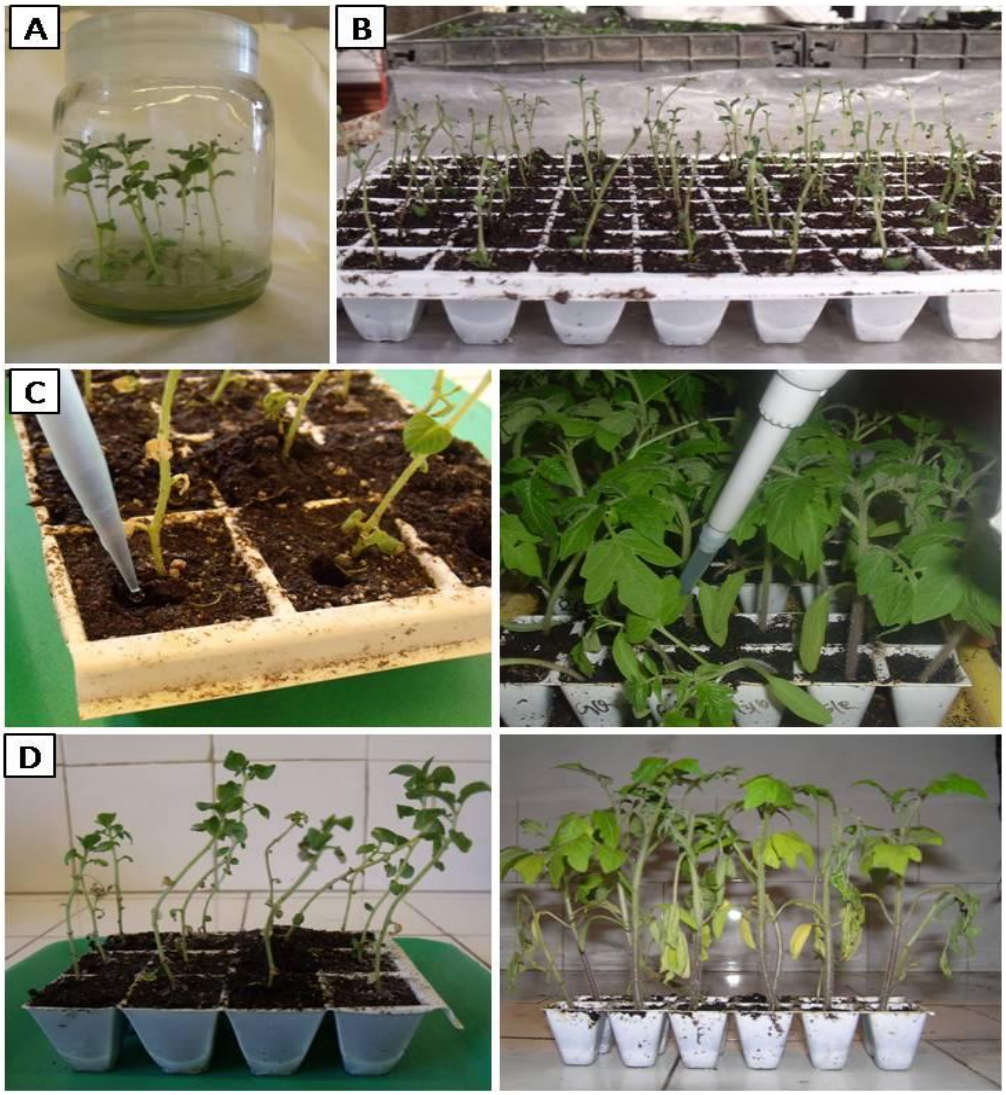


Figure 2-6. Overview of the experimental assays for aggressiveness evaluation of Uruguayan *Ralstonia solanacearum* strains on potato (*Solanum tuberosum* cv. Chieftain) and tomato (*Solanum lycopersicum* cv. Loica). A. Three-weeks old potato plantlets grown in vitro on Murashige and Skoog medium supplemented with 30 g of sucrose per liter. B. Greenhouse acclimatization of potato plants transferred into plastic flats with commercial soil mix. C. Soil inoculation of potato (left) and tomato (right) plants by pouring 1 ml of bacterial suspension into a hole next to the base of each plant (5 x 10^6 cfu/g soi). D. Symptom appearance on potato (left) and tomato (right) plants.

Control plants treated with saline solution remained healthy in all experiments. Based on AUDPC, Uruguayan strains were classified in two groups of aggressiveness, each comprising the same set of strains regardless the host assayed ($p<0.0001$) (Table 2-2). Strains UY031, UY032, UY034, UY036, UY041, UY042, UY072, UY082, UY084, UY085, UY087, UY089, UY091, UY092 and the reference strain UW551, showed high aggressiveness on both hosts. Tomato and potato plants began to develop wilting symptoms 5 to 7 days after inoculation, and more than 80 % of the plants were completely dead at the end of the experiments. In contrast, strains UY033, UY035, UY043 and UY071 exhibited a lower virulence level on both tomato and potato. In this group, onset of symptoms was delayed and a reduced proportion of wilting plants was observed for both hosts (Figure 2-7). Reference strain IPO1609 was grouped with less aggressive Uruguayan's strains on tomato, though displayed an intermediate response on potato. Based on the comparison of AUDPC means either on tomato and potato, no significant differences in aggressiveness were found between groups of strains isolated in different years during the period 2003-2009 or belonging to different potato-growing regions.

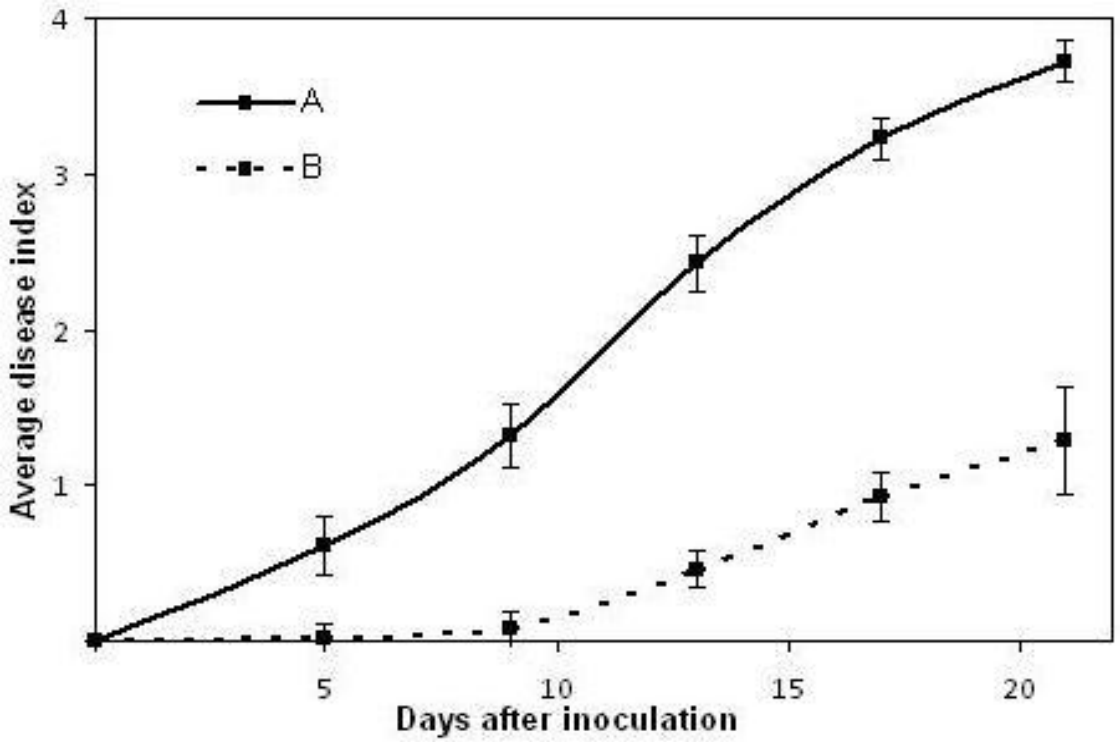


Figure 2-7. Representative patterns of bacterial wilt evolution on potato cv. 'Chieftain' after soil inoculation with Uruguayan *Ralstonia solanacearum* strains with significant differences in aggressiveness (Tukey's multiple comparison test, p = 0.05). Group A include strains: UY031, UY032, UY034, UY036, UY041, UY042, UY082, UY084, UY085, UY087, UY089, UY091, UY092 and reference strain UW551. Group B includes strains: UY033, UY035, UY043 and UY071. Bars represent standard deviation.

Table 2-2. Aggressiveness level of *Ralstonia solanacearum* strains on tomato (*Solanum lycopersicum* cv. Loica) and potato (*Solanum tuberosum* cv. Chiefatin).

Strain	Aggressiveness level (AUDPC [a])	
	Tomato	Potato
UW551	46.2 A	40.8 A
UY031	44.0 A	40.0 A
UY032	36.5 A	39.7 A
UY036	36.1 A	38.7 A
UY041	42.8 A	37.5 A
UY091	ND	34.9 A
UY034	38.7 A	39.2 A
UY085	ND	38.7 A
UY042	42.3 A	38.4 A
UY082	ND	37.6 A
UY084	ND	36.0 A
UY087	ND	36.4 A
UY092	ND	33.4 A
UY089	ND	32.1 A
UY072	42.1 A	ND
IPO1609	20.1B	23.9 AB
UY071	10.5 B	9.3 B
UY035	14.8 B	10.3 B
UY033	15.9 B	7.6 B
UY043	9.3 B	6.4 B

ND: not determined

[a] AUDPC: area under the disease progress curve based on average wilting scoring. Values followed by the same letter in the same column are not significantly different (Tukey's multiple comparison test, p = 0.05).

Variation in aggressiveness among 13 Uruguayan strains was also observed by inoculating three *S. commersonii* accessions carrying different levels of resistance to bacterial wilt (Table 2-3, Figure 2-8). *S. tuberosum* was included in the analysis as a susceptible control, obtaining comparable results with respect to the experiments described above. Analysis of variance resulted in highly significant differences within both strains and hosts ($p<0.0001$). No significant interaction effect was observed between the main factors ($p=0.0763$). Based on AUDPC data, Uruguayan strains were classified in two aggressiveness groups. Strains UY031, UY032, UY036, UY041, UY042, UY072, UY082, UY084, UY087 and UY091 were significantly more aggressive, while strains UY033, UY043 and UY071 displayed a lower level of aggressiveness. In the latter group, UY043 was the less aggressive strain on every host tested, however the differences with other strains in this group were not statistically significant.

S. commersonii accessions differed in the level of resistance after soil inoculation with Uruguayan *R. solanacearum* strains (Table 2-3). Sc19 was the most susceptible accession, showing a similar behavior to *S. tuberosum* for all strains assayed. Sc1 was the most resistant accession followed by Sc26, which displayed an intermediate response. For the latter two genotypes, asymptomatic plants predominated, as well as delayed and slight symptoms of wilting in comparison to the susceptible control *S. tuberosum*. Strains classified as more aggressive were able to wilt at least in some degree, all *S. commersonii* accessions, whereas less aggressive strains failed to cause disease on the most resistant accession Sc1.

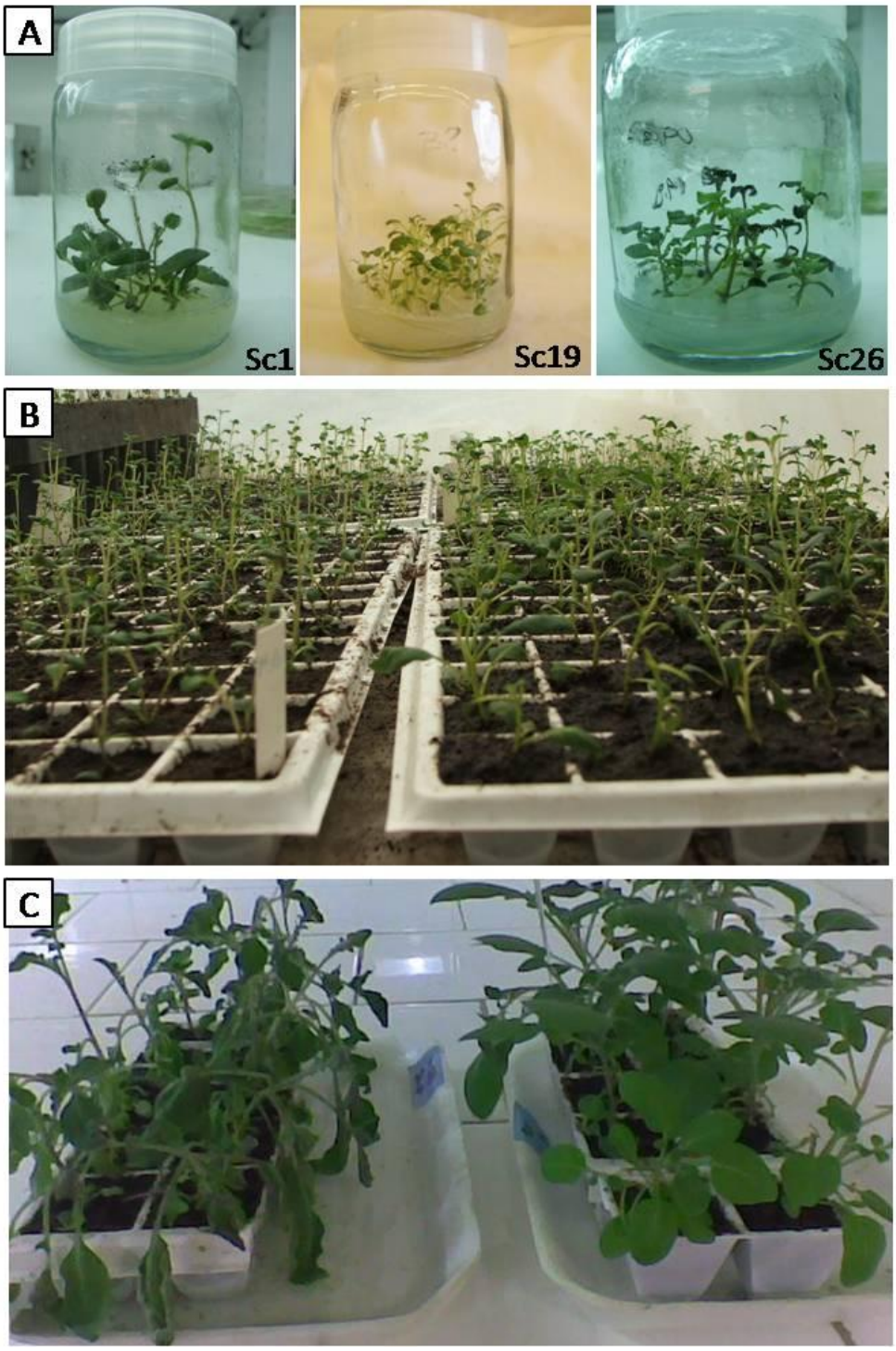


Figure 2-8. Overview of the experimental assay for aggressiveness evaluation of Uruguayan *Ralstonia solanacearum* strains on *Solanum commersonii.* A. Four-weeks old *S. commersonii* plantlets of accessions Sc1, Sc19 and Sc26, grown in vitro on Murashige and Skoog medium supplemented with 30 g of sucrose per liter. B. Greenhouse acclimatization of *S. commersonii* plants transferred into plastic flats with commercial soil mix. C. Symptom appearance on *S. commersonii* (Sc19) inoculated with *R. solanacearum* strain UY031 (left) in comparison with the corresponding negative control inoculated with saline solution (right).

Table 2-3. Aggressiveness level of *Ralstonia solanacearum* strains on potato (*Solanum tuberosum* cv. Chieftain) and different *Solanum commersonii* accessions.

Strain	Aggressiveness level (AUDPC [a])				Mean [b]
	S. tuberosum	*S. commersonii* accessions			
		Sc26	Sc19	Sc1	
UY031	47.2	40.8	23.6	5.3	29.2 A
UY041	47.1	43.0	22.2	3.9	29.0 A
UY091	39.0	42.2	21.7	5.3	27.0 A
UY042	40.5	42.0	21.4	3.5	26.8 A
UY082	41.9	41.4	19.0	3.6	26.5 A
UY036	39.4	40.8	17.4	7.6	26.3 A
UY072	40.2	39.6	22.3	3.3	26.3 A
UY032	41.4	37.5	20.4	5.6	26.2 A
UY087	40.0	36.0	17.7	8.3	25.5 A
UY084	35.8	41.7	22.8	1.4	25.4 A
UY071	22.8	23.4	4.8	0.0	12.7 B
UY033	21.1	21.2	2.7	0.0	11.2 B
UY043	10.8	11.8	0.9	0.0	5.9 B
Mean [b]	35.9 A	35.5 A	16.7 B	3.7 C	

[a] AUDPC: area under the disease progress curve based on average wilt scoring for each host-strain combination. [b] Average AUDPC for each strain and host. Values followed by the same letter in the same column or row are not significantly different (Tukey's multiple comparison test, p = 0.05).

Genetic diversity

BOX-PCR fingerprints were used to analyze genomic variation within *R. solanacearum* strains isolated from potato fields in Uruguay. A reproducible pattern of amplification products was obtained, with 97% as the lowest level of reproducibility between replicates. Banding patterns contained between 17 and 21 bands ranging from 200 to 3000 bp in size. A total of 45 discrete amplified products were scored and 8 (33.4%) were polymorphic in at least one pair-wise comparison between *R. solanacearum* strains classified as phylotype II, sequevar 1. High similarity was found among genetic profiles of Uruguayan *R. solanacearum* strains as well as between Uruguayan and reference strains IPO1609 and UW551. Cluster analysis of fingerprint patterns revealed two groups distinguished at a mean level of similarity of 82% (Figure 2-9). One predominant cluster included reference strains IPO1609 and UW551, all Uruguayan

strains isolated during the period 2003-2009 and 8 strains isolated in the 1980s. The second cluster included strains DGSA119, DGSA127 and DGSA135 isolated during the 1980s. The average similarity between strains in the first and second cluster was 94% and 100% respectively. Strain GMI1000, used as outgroup for the clustering analysis, showed a low level of genetic similarity (45 %) when compared with the remaining strains, thus substantiating the phenetic reliability of the dendogram. Moreover, the analysis of cophenetic correlations resulted in a very high matrix correlation value (r = 0.945) indicating a good level of association of similarity values with the derived clusters of the dendrogram.

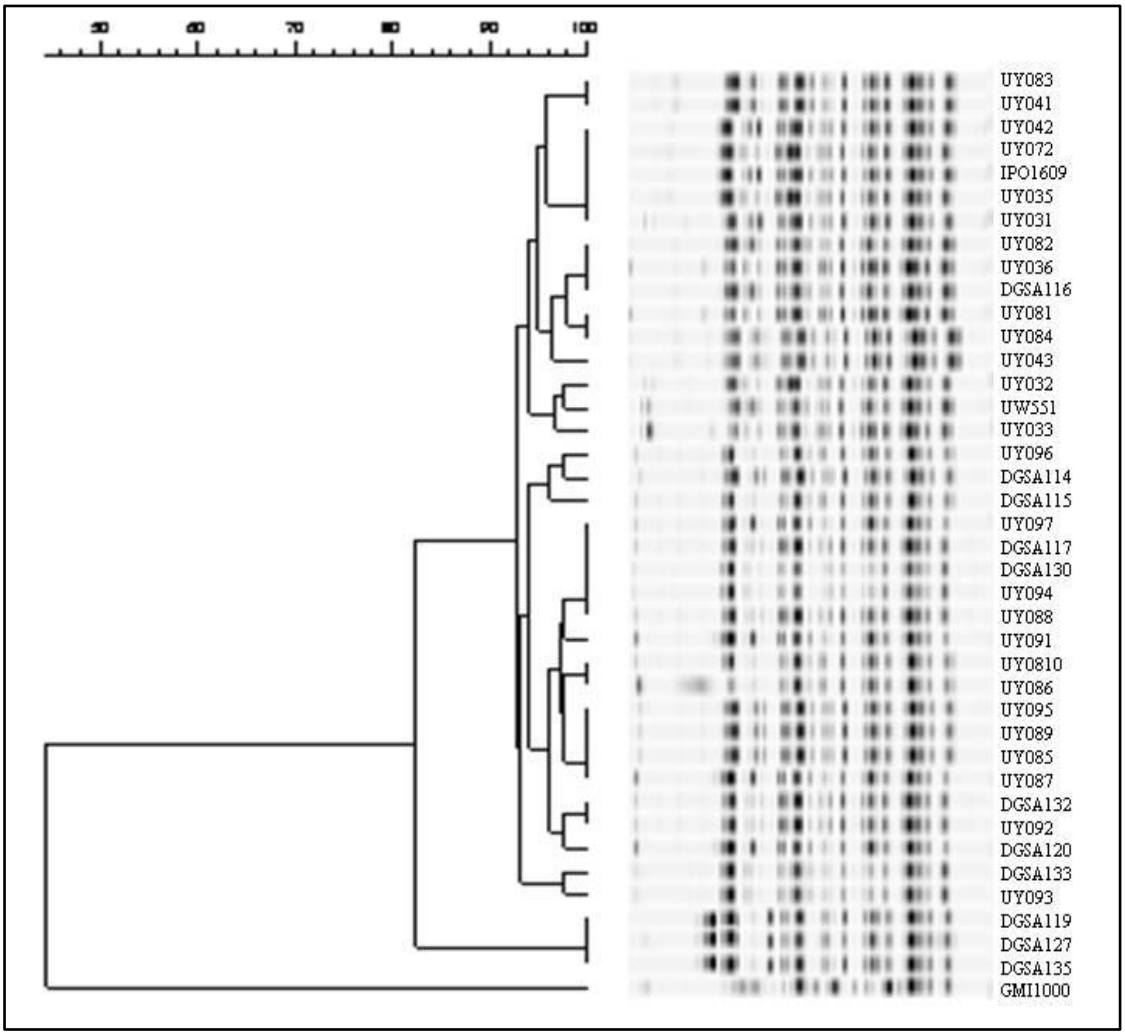


Figure 2-9. Dendrogram based on BOX-PCR fingerprints of 37 *Ralstonia solanacearum* strains from Uruguay created using UPGMA clustering of Dice coefficient values. Reference strains included in the analysis are GMI1000 (outgroup), IPO1609 and UW551.

Comparative genomic hybridization (CGH) analysis

CGH microarray analysis was also applied to identify overall genetic relatedness among *R. solanacearum* strains from Uruguay. The analysis was conducted using a microarray based on the genome sequence of *R. solanacearum* strain GMI1000, complemented with strain-specific genes from strains IPO1609 and Molk2. This technology allowed us to establish the gene content of each tested strain and to perform comparisons on a genome-wide scale. A schematic representation of the different steps involved in CGH analysis is shown in Figure 2-10. The hybridization data and the gene content determined for 15 Uruguayan strains are presented in Appendix 1 and 2.

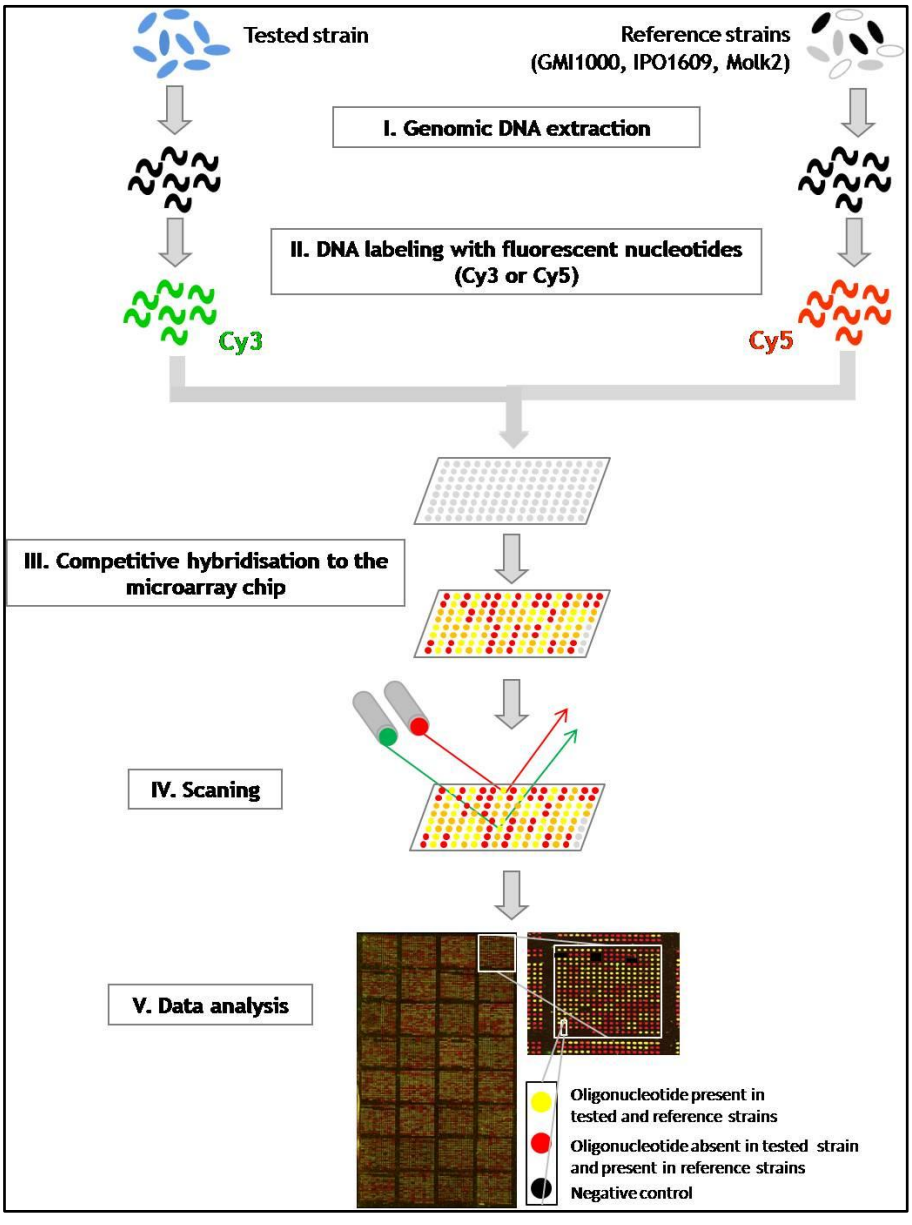


Figure 2-10. Overview of the comparative genomic hybridization (CGH) analysis using a microarray chip containing 6515 oligonucleotides representative of the pan-genome of *Ralstonia solanacearum.* Each experiment consisted of a competitive hybridization between genomic DNA from a tested strain and a standard control DNA consisting of an equimolar combination of the genomic DNA from the 3 sequenced strains GMI1000, IPO1609, and Molk2. Tested and control genomic DNAs were labeled through amplification using random primers and incorporation of fluorescent nucleotides Cy3-dCTP or Cy5-dCTP. Hybridizations were performed by combining equal amounts of tested and control purified fluorescent probes. Following hybridization, microarrays chips were washed to remove non hybridized probes, dried, and scanned using a dual-channel confocal laser scanner at 532 nm (Cy3) and 645 nm (Cy5) excitation wavelengths. Processing, quantification and analysis of the signals emitted at each spot were performed using ImaGene and Genesight software (BioDiscovery, Inc.). The presence or absence of a gene in each tested strain was determined by comparing the ratio of the hybridization signal of the tested strain (green) to that of the reference control strains (red) as described in Materials and Methods.

An overview of the CGH results obtained in this study for 15 Uruguayan *R. solanacearum* strains is shown in Table 2-4. Considering all the 6515 oligonucleotides spotted on the microarray slides, 3643 (55.9%) were identified as present in all strains tested. Many of these oligonucleotides (2177, data not shown) represent genes previously identified as part of the core genome of *R. solanacearum* (Guidot et al., 2007). In addition, a high proportion (78.4%) of the of genes incorporated to the microarray based on the genome sequence of strain IPO1609, were also detected as present in all Uruguayan *R. solanacearum* strains, in agreement with their affiliation to the IIB-1 group. A total of 2858 genes were found to be absent (or too divergent to be detected) in at least one *R. solanacearum* strain from Uruguay. Among these genes, 474 were absent in all tested strains while 2384 showed a variable response. The functional categories of all these variable genes are given in Figure 2-11. A hierarchical clustering was performed to further examine the relation of Uruguayan *R. solanacearum* strains to the whole diversity found in the species complex. For that purpose, we included CGH data for additional *R. solanacearum* strains covering different phylotpes (Figure 2-12). This analysis showed all Uruguayan *R. solanacearum* strains clustered together with other reference IIB-1 strains. A distinct sub-cluster was observed for *R. solanacearum* strains DGSA119, DGSA127 and DGSA135, isolated in the

1980s, demonstrating a strong correlation with the genetic diversity analysis based on BOX-PCR fingerprints (Figures 2-9 and 2-12).

Table 2-4. Overview of the results obtained for the comparative genomic hybridization (CGH) analysis of 15 Uruguayan *Ralstonia solanacearum* strains using a pan-genome microarray representing the genome sequence of three reference strains (GMI1000, IPO1609 and Molk2)[a]

Genes	Spotted	Present	Absent	Variable	Invalid (no data)
GMI1000	5303	2938	354	1998	13
Chromosome	3504	2172	185	1137	10
Megaplasmid	1799	766	169	861	3
IPO1609	709	556	0	153	0
Molk2	490	149	120	220	1
Total	6515	3643	474	2384	14

[a] The lists of present, absent and invalid genes found in all Uruguayan *R. solanacearum* strains were established based on the trinary score determined for each oligonucleotide spotted on the microarray (present = 1; absent = 0; missing data = 2) (see Methods).

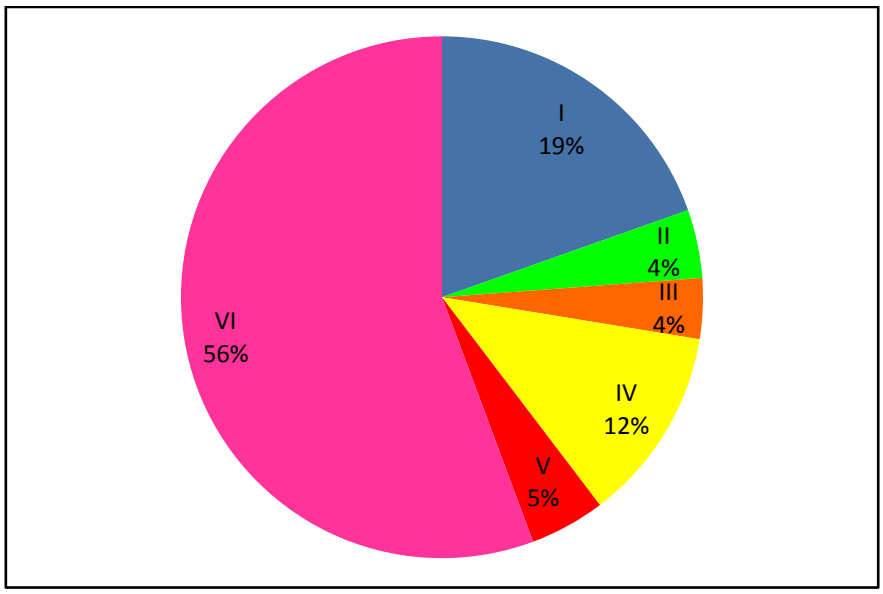


Figure 2-11. Distribution of 2384 variable genes within the different functional categories defined in the genome annotation of *Ralstonia solanacearum* strains GMI1000, IPO1609 and Molk2. I: small molecule metabolism, II: macromolecule metabolism, III: structural elements, IV: cell processes, V: elements of external origin, VI: miscellaneous (http://sequence.toulouse.inra.fr/R.solanacearum)

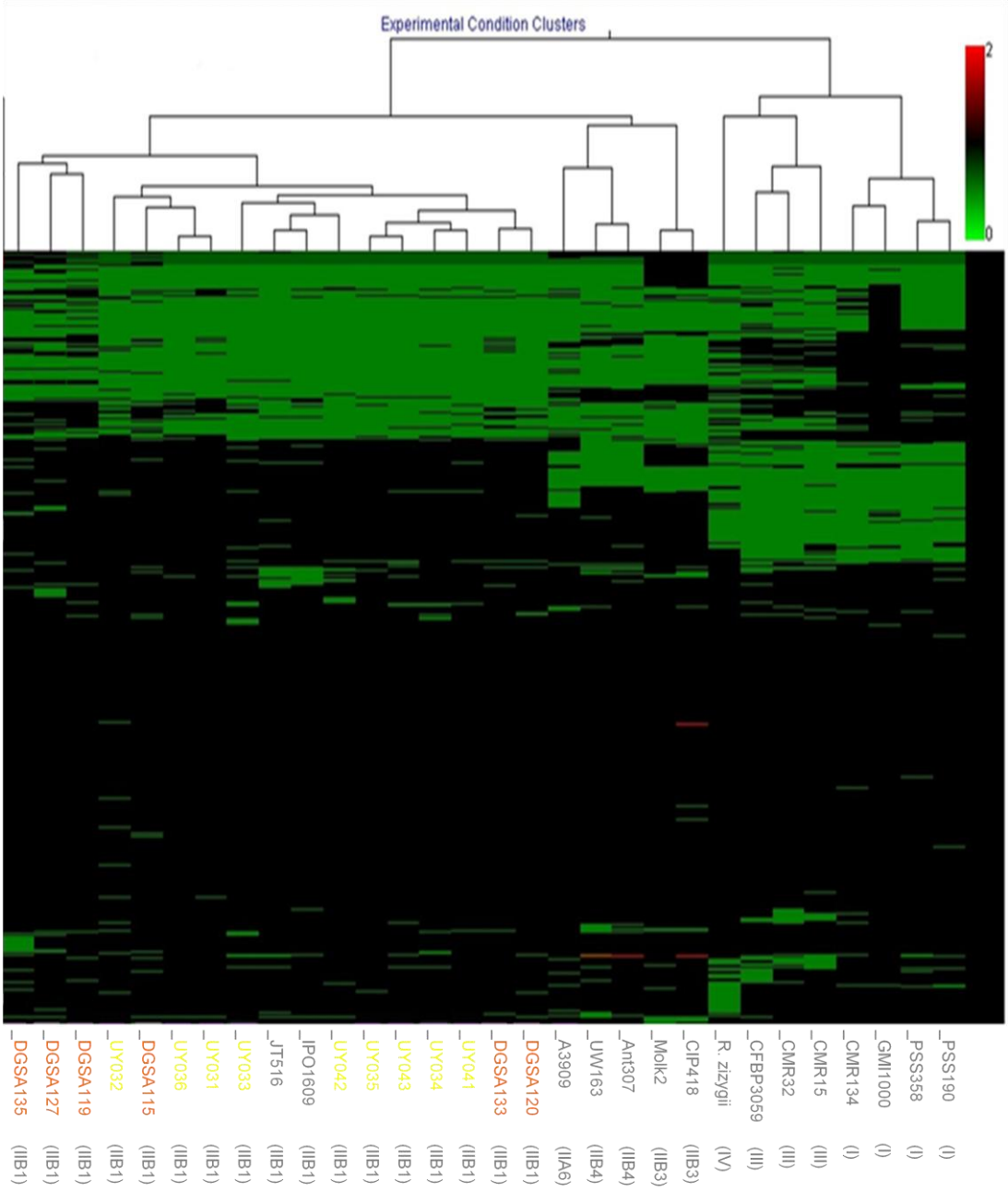


Figure 2-12. Clustering of *Ralstonia solanacearum* strains based on gene distribution determined by microarray comparative genomic hybridization analysis. The gene status based on cut-off values of absence and presence predictions is shown color-coded: black (present); green (absent); red (no data). *R. solanacearum* strains from Uruguay isolated in 2003 and 2004 are (yellow), or in the 1980s (orange) or other strains from distinct origins and phylotypes (grey) are designated vertically across the bottom, together with their respective phylotype affiliation. The Ward technique was used for cluster linkage and the Euclidian method for distance metric using Gensight software (Biodiscovery, Inc.).

DISCUSSION

Bacterial wilt caused by *R. solanacearum* is a major limiting factor for potato production in Uruguay. Severe outbreaks have occurred periodically in all production areas since 1980, but until now there is no published report concerning the identification and characterization of local strains of the pathogen. Knowledge of the variation in the population of the target pathogen forms the basis of epidemiological studies and control measures and will provide us a key factor for the development of resistant potato cultivars. This study involved the analysis of *R. solanacearum* strains affecting potato crops in Uruguay by means of genetic characterization and aggressiveness evaluation on different hosts.

Results of this study suggest low diversity among *R. solanacearum* strains affecting potato crops in Uruguay. Biovar determination showed that all strains were classified as biovar 2. In addition, based on the hierarchical classification system proposed by Fegan and Prior (2005), all strains in our Uruguayan's collection were assigned to the phylotype IIB, sequevar 1 (IIB-1), historically known as race 3 biovar 2. This finding is in agreement with previous reports stating that IIB-1 strains are adapted to mild temperatures and therefore, constitute a serious threat to agricultural production in temperate regions of the world (Lambert, 2002; Van Elsas et al., 2000; Williamson et al., 2002). Strains belonging to race 1 have also been detected in Uruguay in the past, mainly affecting tomato crops in the north of the country (E. Verdier, personal communication). However, sanitary measures were taken for eradication, and at the moment this type of strains is considered a quarantine pest in Uruguay. Race 1 strains can affect a wide range of host plants in contrast to race 3 that have a relative narrow host range (Hayward, 1994b). Furthermore, race 1 strains have been reported in southern Brazil (Silveira et al., 1998), meaning an ongoing risk of introduction through bordering areas where exchange of plant material is hard to control. Therefore, research and monitoring bacterial wilt in different potential hosts should be regularly implemented, and governmental control of potato and plant material flows at the borders should be strict.

In this research we also included a set of *R. solanacearum* strains isolated in the 1980s and maintained in the collection of the governmental Plant Protection Service in Uruguay. These archival strains were non pathogenic on potato after direct stem inoculation so they were not included in the comparative aggressiveness analysis. This finding is in accordance with the particular phenotype of these strains in TZC medium, which resembled phenotypic conversion mutant strains previously described in *R. solanacearum* (Kelman, 1954). Spontaneous phenotypic conversion mutants are generated in some stressful conditions, and they are often observed under long term storage or repetitive sub-culturing. When this phenomenon occurs, the wild-type strain undergoes loss of pathogenicity associated with multiple changes including colony morphology, motility and production of virulence factors (Genin et al., 2005; Kelman, 1954; Schell, 2000). It was established that susceptible crops can specifically induce reversion from phenotype conversion to wild-type (Poussier et al., 2005). However, we did not manage to re-isolate any presumptive bacteria from asymptomatic plants 14 days after inoculation with these strains. Despite its particular phenotype, we performed the molecular characterization of all these strains, revealing that belonged to the IIB-1 group. These results are in agreement with its previous assignation as race 3, biovar 2.

Variation in aggressiveness between Uruguayan *R. solanacearum* strains isolated during the period 2003-2009 was detected on several hosts after soil inoculation. Assays were performed on tomato and potato since these are the main susceptible crops in Uruguay. Most Uruguayan strains showed a similar behavior to the reference strain UW551, displaying high virulence on both hosts. In contrast, some other strains exhibited lower virulence level, with typical delay in the appearance of symptoms and a significantly high proportion of non-wilted plants at the end of the experiments. These strains were found to be less aggressive on both hosts and in repeated experiments, supporting this finding. Several studies reported differences in aggressiveness between *R. solanacearum* strains belonging to others biovars or comparing different biovars and races (Fock et al., 2005; Jaunet and Wang, 1999; Lopes et al., 2005). However, to our knowledge, this is the first report of different

aggressiveness levels among *R. solanacearum* potato brown rot strains belonging to the IIB-1 group.

No significant differences in aggressiveness were found between groups of strains with different geographic origin, indicating that this feature is not associated with a particular outbreak or production region. Because only two strains isolated from soil were evaluated in this study (UY043 and UY071), no conclusions can be made regarding the nature of the samples used for isolation of *R. solanacearum* strains (soil or tuber). Recently, it was reported the first genotypic and phenotypic characterization of a set of environmental *R. solanacearum* biovar 2 strains (Stevens and Van Elsas, 2009). In this study, no differences in pathogenicity were found between environmental and potato-derived strains. However, this evaluation was done with a low number of strains and limited to tomato, not allowing to a clear conclusion regarding the pathogenic fitness of environmental isolates (Stevens and Van Elsas, 2009). Due to the limited data available and the low aggressiveness observed for the two strains isolated from soil, further research comparing pathogenicity of environmental and plant derived *R. solanacearum* strains is warranted.

Aggressiveness of *R. solanacearum* strains was also evaluated on *S. commersonii*, a wild tuber-bearing species native to Uruguay. Some studies have reported the utilization of *S. commersonii* as a source of resistance to bacterial wilt (Carputo et al., 2009; Laferriere et al., 1999). However, little is known about resistance properties across the whole spectrum of diversity in this wild species. In the present study, three *S. commersonii* genotypes were selected from a previous screening (Siri et al., 2009) and tested against a subset of *R. solanacearum* strains. No significant interaction was found between strains and genotypes, since all strains were ranked in the same order regardless the *S. commersonii* accession tested. We found differences in aggressiveness among *R. solanacearum* strains, consistently with aggressiveness groups based on tomato and potato experiments. These findings suggest that low aggressiveness observed for some strains is not host-specific. Moreover, these results indicate that germplasm selection with high aggressive strain seems to be a promising strategy for breeding programs in Uruguay.

Regarding the host, different levels of resistance between *S. commersonii* accessions were found. Variation in resistance among *S. commersonii* is in agreement with our previous screening using a direct stem inoculation procedure (Siri et al., 2009). This similar response using such different inoculation methods may indicate that resistance in *S. commersonii* did not result from unsuccessful root colonization, but is rather associated with the ability of the plant to restrict bacterial invasiveness and multiplication inside the plant tissues. Unfortunately, little is known on the plant–pathogen interaction in potato. Studies on tomato suggest that activation of secondary metabolism of phenolic compounds and xylem cell wall structure appear to contribute to control bacterial development into the plant (Vasse et al., 2005; Wydra and Ber, 2007). Further research comparing root infection process of *S. commersonii* accessions with different levels of resistance will be conducted in order to test this hypothesis.

Accession Sc1 was consistently the most resistant genotype, showing mild symptoms even when challenged with most aggressive strains. Based on these results, this accession was therefore selected as a progenitor in the potato breeding program which is in progress in Uruguay. Studies are now underway to investigate the genetic basis of this resistance, through the development of a segregating population from the crossing of different *S. commersonii* accessions. Characterization of bacterial wilt resistance in these target populations revealed high segregation of this character, suggesting that resistance in *S. commersonii* is determined by few independent genes with additive effect (M. González, personal communication). Overall, this study highlights the potential of *S. commersonii* as a useful source of resistance for potato breeding. This wild species harbors a great amount of genetic and phenotypic diversity and is specially adapted to our environmental conditions. Results obtained in this study confirmed the existence of different levels of resistance within this wild species and helped us to identify the most promising genotypes for breeding purposes. It is not expected that resistance alone will solve the bacterial wilt problem in potato crops, although it might contribute to an integrated approach to control the disease in Uruguay.

In this research, we also evaluated the genetic diversity of Uruguayan *R. solanacearum* strains by BOX-PCR and CGH using a microarray chip reflecting the pan-genome of *R.*

solanacearum. Overall, both analysis revealed low genetic variability among Uruguayan strains and confirmed their classification as phylotype IIB, sequevar 1.

DNA fingerprints analysis based on rep-PCR have been widely used to assess diversity and genetic relationships among *R. solanacearum* strains (Horita et al., 2010; Jaunet and Wang, 1999, Lewis et al., 2007; Smith et al., 1995; Van der Wolf et al., 1998). PCR-based techniques are usually subjected to variations that reflect many factors, such as template and primer DNA concentrations and composition of the PCR buffer. Therefore, we compared the band patterns between replicates and we found a high level of reproducibility (97%) under our experimental conditions. High similarity was found among genetic profiles of Uruguayan *R. solanacearum* strains, suggesting that a signal clonal population could be involved in outbreaks of bacterial wilt in all potato-growing areas in Uruguay. Despite the low genetic variability, clustering analysis of BOX fingerprints patterns revealed two distinct groups. The major or predominant cluster contained most of the Uruguayan isolates as well as reference strains (IPO1609 and UW551), in agreement with the phylogenetic assignation of Uruguayan isolates. It is noteworthy that certain archival isolates collected from potato crops during the 1980s had fingerprints almost equal to those of the contemporary strains, suggesting the prevalence of the same genetic lineage along this period. On the other hand, three strains isolated during the 1980s in the same location (San José) showed some extent of genetic variation. Indeed, these isolates formed a separate group with 84% similarity respect to the remaining isolates.

Several previous studies revealed low genetic variation among IIB-1 *R. solanacearum* strains using fingerprinting genetic markers and sequence analysis of specific genes (Castillo and Greenberg, 2007; Poussier et al., 2000; Timms-Wilson et al., 2001; Van der Wolf et al., 1998). In support, the draft genomes of two 2 strains from this group (UW551 and IPO1609) are now available revealing almost an identical sequence and genome structure (Gabriel et al., 2004; Guidot et al., 2009). DNA microarray-based CGH has been used to explore genetic diversity in several different microbial pathogens (Betancor et al., 2009; Guidot et al., 2007; He et al., 2007; Lindsay and Holden, 2006; Quiñones et al., 2008). A microarray representing the whole genome of the first sequenced *R. solanacearum* strain, GMI1000, was previously used for CGH

analysis of 18 strains from different phylotypes (Guidot et al., 2007). Using this approach, the authors were able to identify a set of 2690 genes conserved in all tested strains, which represent the core genome of *R. solanacearum.* In addition, they also identified a repertoire of variable genes representing 46% of the GMI1000 genome, which are typically clustered in genomic islands probably acquired by horizontal gene transfers (Guidot et al., 2007). Recently, this DNA microarray was complemented with specific genes identified from the genome sequence of two additional *R. solanacearum* strains belonging to the phylotype II: IPO1609 (IIB-1) and Molk2 (IIB-3). To further explore the genomic variation among Uruguayan *R. solanacearum* strains, we conducted a CGH analysis using this pan-genome microarray. As expected, the gene composition determined for all Uruguayan *R. solanacearum* strains resembled that of reference strain IPO1609, thereby confirming their assignation to the IIB-1 group. Despite the high degree of genetic homogeneity observed for Uruguayan *R. solanacearum* strains, the CGH approach used in this study detected a total of 2384 genes with a variable distribution among the tested strains. It is noteworthy that more than half of these genes have been predicted to encode proteins with unknown functions, highlighting the need to give a biological meaning to the available sequence information. Hierarchical clustering based on CGH data including additional *R. solanacearum* strains from distinct origins and pylotypes, generated a dendrogram congruent with the validated phylogeny of the species complex (Fegan and Prior, 2005). This concordance is in agreement with a previous analysis performed using CGH microarray based only on the GMI1000 genome (Guidot et al., 2007). Accordingly, in this study all Uruguayan strains were clustered together with other IIB-1 strains. Interestingly, a strong correlation was observed between hierarchical clustering based on BOX-PCR fingerprints and CGH analysis, since both analysis identified a distinct sub-cluster for the same 3 *R. solanacearum* strains isolated in the 1980s. This finding resulted from such different molecular approaches, revealed that these strains harbor a distinct genotype which is not represented in the contemporary strains, suggesting that it was lost during this period.

In summary, the CGH analysis performed in this study allowed us to establish the genetic content of each tested strain. This information together with the

aggressiveness characterization of Uruguayan *R. solanacearum* strains, pave the way toward identifying pathogenicity correlations and developing improved epidemiological tools to control this problematic pathogen. These approaches will be discussed in Chapters 3 and 4 of the present thesis.

REFERENCES

Ausubel F.H., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., and Struth K. 1994. Current Protocols in Molecular Biology. John Wiley and Sons, Canada.

Betancor L., Yim L., Fookes M., Martinez A., Thomson N.R., Ivens A., Peters S., Bryant C., Algorta G., Kariuki S., Schelotto F., Maskell D., Dougan G., and Chabalgoity J.A. 2009. Genomic and phenotypic variation in epidemic-spanning *Salmonella enterica* serovar Enteritidis isolates. BMC Microbiol. 9:237.

Boshou L. 2005. A broad review and perspective on breeding for resistance to bacterial wilt. 2005. Pages 225-238 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A. C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Bradshaw J.E., Bryan G.J., and Ramsay G. 2006. Genetic resources (including wild and cultivated *Solanum* species) and progress in their utilisation in potato breeding. Potato Res. 49**:**49-65.

Buddenhagen I., Sequeira L., and Kelman A. 1962. Designation of races in *Pseudomonas solanacearum*. Phytopathology 52:726.

Carputo D., Castaldi L., Caruso I., Aversano R., Monti L., and Frusciante L. 2007. Resistance to frost and tuber soft rot in near-pentaploid *Solanum tuberosum–S. commersonii* hybrids. Breeding Sci. 57:145–151.

Carputo D., Aversano R., Barone A., Di Matteo A., Iorizzo M., Sigillo L., Zoina A., and Frusciante L. 2009. Resistance to *Ralstonia solanacearum* of sexual hybrids between *Solanum commersonii* and *S. tuberosum*. Am. J. Pot Res. 86:196–202.

Denny T.P. 2006. Plant pathogenic *Ralstonia* species. Pages 573-644. in: Plant-Associated Bacteria. S.S. Gnanamanickam ed. Springer Publishing, Dordrecht, The Netherlands.

Di Rienzo J.A., Casanoves F., Balzarini M.G., Gonzalez L., Tablada M., and Robledo C.W. InfoStat versión 2009. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

Elphinstone J.G. 2005. The current bacterial wilt situation: a global overview. Pages 9-28 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A.C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Elphinstone J.G., Hennessey J., Wilson J.K., and Stead D.E. 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. EPPO Bulletin 26:663–78.

Fegan M., Taghavi M., Sly L.I., and Hayward A.C. 1998. Phylogeny, diversity and molecular diagnostics of *Ralstonia solanacearum*. Pages 19-33 in: Bacterial Wilt disease: Molecular and Ecological Aspects. P. Prior, C. Allen, and J. Elphinstone, eds. Springer-Verlag, New York.

Fegan M. and Prior P. 2005. How complex is the '*Ralstonia solanacearum*' species complex? Pages 449- 461 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A. C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Fock I., Luisetti J., Collonier C., Vedel F., Ducreux G., Kodja H., and Sihachakr D. 2005. *Solanum phureja* and *S. stentotum* are sources of resistance to *Ralstonia solanacearum* for

somatic hybrids of potato. Pages 253-259 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A.C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

French E.R., Anguiz R., and Aley F.P. 1998. The usefulness of potato resistance to *Ralstonia solanacearum* for the integrated control of bacterial wilt. Pages: 381-385 in: Bacterial Wilt disease: Molecular and Ecological Aspects. P. Prior, C. Allen, and J. Elphinstone, eds. Springer-Verlag, New York.

Genin S., Brito B., Denny T.P., and Boucher C. 2005. Control of the *Ralstonia solanacearum* type III secretion system (Hrp) genes by the global virulence regulator PhcA. FEBS Letters 579:2077-2081.

Guidot A., Prior P., Schoenfeld J., Carrere S., Genin S., Boucher C.A. 2007. Genomic structure and phylogeny of the plant pathogen *Ralstonia solanacearum* inferred from gene distribution analysis. J. Bacteriol. 189:377–387.

Guidot A., Elbaz M., Carrere S., Siri M.I., Pianzzola M.J., Prior P. and Boucher C. 2009. Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathology 99:1105–1112.

Hayward A.C. 1964. Characteristcs of *Pseudomonas solanacearum.* Journal of Appl. Bacteriol. 27:265–77.

Hayward A.C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum.* Annu. Rev. Phytopathol. 29:65–87.

Hayward A.C. 1994a. Systematics and phylogeny of *Pseudomonas solanacearum* and related bacteria. Pages 123-135 in: Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A.C. Hayward and G.L. Hartman, eds. CAB International, Wallingford, UK.

Hayward A.C. 1994b. The hosts of *Pseudomonas solanacearum*. Pages 9-23 in: Bacterial wilt: The disease and its causative agent, *Pseudomonas solanacearum.* A.C. Hayward and G.L. Hartman, eds. CAB International Wallingford, UK.

He Y.L., Sequeira L., and Kelman A. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Dis. 67:1357-1361.

He Y.Q., Zhang L., Jiang B.L., Zhang Z.C., Xu R.Q., Tang D.J., Qin J., Jiang W., Zhang X., Liao J., Cao J.R., Zhang S.S., Liang X.X., Wei M.L., Lu G.T., Feng J.X., Chen B., Cheng J., and Tang J.L.. 2007. Comparative and functional genomics reveals genetic diversity and determinants of host specificity among reference strains and a large collection of Chinese isolates of the phytopathogen *Xanthomonas campestris* pv. *campestris*. Genome Biol. 8(10):R218.

Hendrick C. and Sequeira L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. Appl. Environ. Microbiol. 48:94–101.

Horita M., Suga Y., Ooshiro A., and Tsuchiya K. 2010. Analysis of genetic and biological characters of Japanese potato strains of *Ralstonia solanacearum.* J. Gen. Plant Pathol. 76:196–207.

Jaunet T.X. and Wang J.F. 1999. Variation in genotype and aggressiveness diversity of *Ralstonia solanacearum* race 1 isolated from tomato in Taiwan. Phytopathology 9:320–327.

Jukes T.H. and Cantor C.R. 1969. Evolution of protein molecules. Pages 121-132 in: Mammalian protein metabolism. H.N. Munro, ed., New York.

Kelman A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693–695.

Kumar S., Tamura K., and Nei M. 2004. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform. 5:150–163.

Laferriere L.T., Helgeson J.P., and Allen C. 1999. Fertile *S.tuberosum + S. commersonii* somatic hybrids as sources of resistance to bacterial wilt caused by *R. solanacearum*. Theor. Appl. Genet. 98:1272–1278.

Lambert C.D. 2002. Agricultural bioterrorism protection act of 2002: Possession, use, and transfer of biological; agents and toxins; interim and final rule (7 CFR Part 331). Federal Register 67:76908-76938.

Lewis Ivey M.L., McSpadden Gardener B.B., Opina N., and Miller S.A. 2007. Diversity of *Ralstonia solanacearum* infecting eggplant in the Philippines. Phytopathology 97:1467–1475.

Lindsay J.A. and Holden M.T. 2006. Understanding the rise of the superbug: investigation of the evolution and genomic variation of *Staphylococcus aureus*. Funct. Integr. Genomics 6:186–201.

Lopes C.A., Carvalho S.I.C., and Boiteux L.S. 2005. Search for resistance to bacterial wilt in a Brazilian *Capsicum* germplasm collection. Pages 247-251 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A.C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Louws F.J., Fulbright D.W., and de Bruijn F.J. 1994. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Appl. Environ. Microbiol. 60:2286-2295.

Montanelli C., Chiari A., Chiari T., Stefanini F., and Nascari G. 1995. Evaluation of resistance to *Pseudomonas solanacearum* in potato under controlled conditions. Euphytica81:35-43.

Montgometry D.C. 2005. Diseño y análisis de experimentos. Editorial Limusa, México.

Occhialini A., Cunnac S., Reymond N., Genin S., and Boucher C.A. 2005. Genomewide analysis of gene expression in *Ralstonia solanacearum* reveals that the *hrpB* gene acts as a regulatory switch controlling multiple virulence pathways. Mol. Plant-Microbe. Interact. 18:938–949.

Pastrik K.H. and Maiss E. 2000. Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. J. Phytopathol. 148:619– 626.

Pegg K. and Moffett M. 1971. Host range of the ginger strain *of Pseudomonas solanacearum* in Queensland. Aust. J. Exp. Agric. Anim. Husb. 11:696-698.

Poussier S., Trigalet-Demery D., Vandewalle P., Goffinet B., Luisetti J., and Trigalet A. 2000. Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the hrp gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. Microbiology 146:1679–1692.

Poussier S., Trigalet-Demery D., Barthet S., Thoquet P., Arlat M., and Trigalet A. 2005. Phase revertion from phenotype conversion mutants to wild type may be induced in *Ralstonia solanacearum* by a susceptible host plant. Pages 351-357 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A.C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Quiñones B., Guilhabert M.R., Miller W.G., Mandrell R.E., Lastovica A.J., and Parker C.T. 2008. Comparative genomic analysis of clinical strains of *Campylobacter jejuni* from South Africa. PLoS ONE 3(4): e2015.

Schell M.A. 2000. Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory array. Annu. Rev. Phytopathol. 38:263-292.

Seal S.E., Jackson L.A., Young J.P.W., and Daniels M.J. 1993. Differentiation of *Pseudomonas solanacearum, Pseudomonas syzygii, Pseudomonas picketti* and the blood disease bacterium by partial 16S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. J. Gen. Microbiol. 139:1587-1594.

Silveira J.R.P., Duarte V., and Moraes M.G. 2002. Ocorrência das biovares 1 e 2 de *Ralstonia solanacearum* em lavouras de batata no Estado do Rio Grande do Sul. Fitopatologia Brasileira 27:450-453.

Silveira E.B., Gomes A.M.A., Michereff S.J., and Mariano R.L.R. 1998. Variability of *Ralstonia solanacearum* populations causing wilt on tomato in Agreste of Pernambuco, Brazil. Bacterial Wilt Newsl. 15:8–10.

Siri M. I., Galván G., Quirici L., Silvera E., Villanueva P., Ferreira F., Franco-Fraguas L., and Pianzzola M.J. 2009. Molecular marker diversity and bacterial wilt resistance in wild *Solanum commersonii* accessions from Uruguay. Euphytica 165:371-382.

Smith J.J., Offord L.C., Holderness M., and Saddler G.S. 1995. Genetic diversity of *Burkholderia solanacearum* (synonym *Pseudomonas solanacearum*) race 3 in Kenya. Appl. Environ. Microbiol. 61:4263-4268.

Stevens P. and Van Elsas J.D. 2009. Genetic and phenotypic diversity of *Ralstonia solanacearum* biovar 2 strains obtained from Dutch waterways. Antonie van Leeuwenhoek 97:171–188.

Tozzini A.C., Cerioani M.F., Saladrigas M.V., and Hopp H.E. 1991. Extreme resistance to infection by potato virus in genotypes of wild tuber-bearing *Solanum* species. Potato Res. 34:317–324.

Van der Wolf J.M., Bonants P.J.M., Smith J.J., Hagenaar M., Nijhuis E., Van Beckhoven J.R.C.M., Saddler G.S., Trigalet A., and Feuillade R. 1998. Genetic diversity of *Ralstonia solancaerum* race 3 in Western Europe determined by AFLP, RC-PFGE and rep-PCR. Pages 44-49 in: Bacterial Wilt disease: Molecular and Ecological Aspects. P. Prior, C. Allen, and J. Elphinstone, eds. Springer-Verlag, New York.

Van Elsas J.D., Kastelein J., Van Bekkum P., Van der Wolf J.M., de Vries P.M., and Van Overbeek L.S. 2000. Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathology 90:1358-1366.

Vasse J., Danoun S., and Trigalet A. 2005. Microscopic studies of root infection in resistant tomato cultivar Hawaii7996. Pages 285-291 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A.C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Williamson L., Nakaho K., Hudelson B., and Allen C. 2002. *Ralstonia solanacearum* race 3, biovar 2 strains isolated from geranium pathogenic on potato. Plant Dis. 86:987-991.

Winstead N.N. and Kelman A. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology 42:628-634.

Wydra K. and Ber. H. 2007. Immunohistochemical changes in methyl-ester distribution of homogalacturonan and side chain composition of rhamnogalacturonan I as possible components of basal resistance in tomato inoculated with *Ralstonia solanacearum*. Physiol. Mol. Plant P. 70:13–24.

CAPÍTULO 3

Análisis genómico comparativo de cepas Uruguayas de *Ralstonia solanacearum* enfocado a la identificación de genes candidatos de virulencia

RESUMEN

Ralstonia solanacearum es el agente causal de la marchitez bacteriana, una de las enfermedades bacterianas más devastadoras del mundo, y reconocido como un microorganismo modelo para el análisis de los mecanismos de patogenia. Este estudio se centra en la virulencia de *R. solanacearum*, en particular en lo que respecta a las cepas pertenecientes al filotipo IIB, secuevar 1 (IIB-1) durante su interacción con la papa como su hospedero natural. Como punto de partida nos basamos en la existencia de diferentes niveles de agresividad en una colección de cepas Uruguayas de *R. solanacearum.* Sobre estas cepas se realizó un análisis de hibridación genómica comparativa (CGH) utilizando un chip microarrays que representa el pan-genoma de 3 cepas secuenciadas de *R. solanacearum.* A través de este análisis se estableció el repertorio de genes presentes en cada cepa, los cuales se utilizaron para realizar comparaciones entre cepas con diferente agresividad u origen de aislamiento. Este enfoque nos permitió identificar la deleción de una región de 33.7-kb en una cepa aislada de suelo y que presenta un nivel de agresividad muy bajo (UY043). El análisis de esta región, denominada DR043, sugiere que este rearreglo genómico está asociado con la baja agresividad de esta cepa, y ofrece la posibilidad de identificar genes candidatos virulencia. Entre los genes presentes en DR043, se encuentra un grupo de seis genes implicados en la biogénesis de fimbrias de tipo IV (Tfp). El análisis funcional realizado sugiere que las pilinas codificadas por estos genes contribuyen a la virulencia de *R. solanacearum*, y a la capacidad de colonización de raíces de papa. Sin embargo, estas proteínas no son esenciales para la movilidad tipo *twitching* en cultivo. Los resultados generados en este estudio también sugieren que existen otros genes dentro de la región DR043 que podrían contribuir a explicar la baja agresividad observada para la cepa UY043, entre los que se destacan dos proteínas efectoras secretadas por el sistema de secreción de tipo III. Por último, este estudio destaca la presencia de reordenamientos genómicos en *R. solanacearum*, que pueden jugar un papel importante en la evolución y la diversificación de la especie.

CHAPTER 3

Comparative genomic analysis of *Ralstonia solanacearum* strains from Uruguay focused on the identification of candidate virulence genes

ABSTRACT

Ralstonia solanacearum is the causative agent of bacterial wilt, one of the most devastating bacterial plant diseases in the world, actually recognized as one of the leading models in the analysis of plant pathogenicity. This study provides insights into the pathogenesis of *R. solanacearum*, in particular with regards to strains belonging to phylotype IIB, sequevar 1 (IIB-1), and their interaction with potato as its natural host. As a starting point we rely on the existence of significant differences in aggressiveness among a collection of *R. solanacearum* strains isolated from potato fields in Uruguay. We conducted a comparative genomic hybridization (CGH) analysis using a microarray chip reflecting the pan-genome of 3 sequenced *R. solanacearum* strains, in order to establish the gene repertoire of each Uruguayan strain and perform comparisons between strains with differences in aggressiveness or source of isolation. This approach allowed us to identify the occurrence of a deletion of a 33.7-kb region in an environmental strain with strongly lower aggressiveness (UY043). Analysis of this region, denoted as DR043, suggested that this genomic rearrangement is associated with low aggressiveness found in this strain and offers potential to identify candidate virulence genes. A cluster of 6 genes involved in type IV pili (Tfp) biogenesis was identified within this region. Functional analysis performed suggests that Tfp-related proteins encoded by this genetic cluster mainly contribute to the early bacterial wilt pathogenesis and colonization fitness of potato roots, although these proteins appeared to be not essential for twitching motility in culture. Results generated in this study also suggest that additional genes located within the region DR043 are involved in the low aggressiveness observed for strain UY043, including 2 genes encoding for effector proteins secreted through the type III secretions system. Further research with the identified candidate genes will attempt to more precisely establish this hypothesis. Furthermore, this study highlights the occurrence of genomic rearrangements in *R. solanacearum*, which are known to play an important role in the evolution and diversification of the species.

INTRODUCTION

Bacterial pathogenesis is the result of dynamic interactions between gene products of both host and pathogen. To understand how pathogenic bacteria interact with their hosts to produce disease is a fundamental issue. In essence, the ability to cause disease in a susceptible host is determined by multiple virulence factors acting individually or together at different stages of infection. Such virulence factors are involved in direct interactions with host tissues, allow the pathogen to avoid recognition, combat plant defense responses, or facilitate access to the nutrients provided by the plant cells.

Ralstonia solanacearum is a gram-negative soil-borne β-proteobacterium that causes bacterial wilt, one of the most devastating bacterial plant diseases in the world. The high importance of this organism results from its worldwide geographical distribution and its wide host range affecting more than 200 plant species, including important food crops such as tomato, potato, banana and eggplant (Elphinstone, 2005; Hayward, 1994b). *R. solanacearum* is a highly diversified pathogen, with exceptional diversity among strains regarding host range, geographical distribution, pathogenicity, epidemiological relationships, and physiological properties (Buddenhagen et al., 1962; Castillo and Greenberg, 2007; Hayward 1964, 1991, 1994a). Actually, it is considered a species complex, a heterogeneous group of related strains which are distributed in four lineages termed phylotypes, defined as monophyletic clusters of strains revealed using phylogenetic analysis of sequence data (Fegan and Prior, 2005). Due to this particular features *R. solanacearum* is actually recognized as one of the leading models in the analysis of plant pathogenicity.

In the natural infection process, *R. solanacearum* invades plants via wounds or secondary root emergence points and propagates to the root cortex and then to the vascular system (Vasse et al., 1995). After the bacterium enters into the xylem, the infection becomes systemic, with further bacterial multiplication and the production of large amounts of extracellular polysaccharides which act to plug vascular bundles therefore leading to complete wilting and eventually plant death (Buddenhagen and

Kelman, 1964). The bacterium then returns to the soil, where it can be associated with plant debris and weed rhizosphere and survive under humid conditions over a long time (Coutinho, 2005). Survival in surface waters has also been shown to play an importante role in the ecology of the bacterium (Van Elsas et al., 2001). A large battery of specialized gene products is required to accomplish tasks at different steps of the infection process (Schell, 2000; Genin and Boucher, 2004). Virulence factors, in addition to EPS production, include various plant cell-wall-degrading enzymes and a plethora of effectors proteins translocated into the plant cell through the type III secretion system (Arlat et al., 1992; Saile et al., 1997; González and Allen, 2003; Liu et al., 2005). Bacterial motility, both including twitching motility mediated by type IV pili, and flagellar swimming motility also contribute to virulence (Liu et al., 2001; Tans-Kersten et al., 2001). However, our knowledge on the complex and apparently highly coordinated pathogenesis of this bacterium is far from sufficient. In addition, to date most of the studies regarding virulence traits of *R. solanacearum* were focused on the interaction with tomato as crop host and *Arabidopsis* as a model organism, while other important susceptible crops such as potato remain to be explored.

The continuing reports of complete genome sequences for a variety of bacteria have fuelled the rapid developments in microbial genomic. For *R. solanacearum* the sequencing and annotation of the complete genome from strain GMI1000 in 2002 has been a major achievement which has contributed to the development of genomic resources to study the pathogenicity determinants in this bacterium (Salanoubat et al., 2002). GMI1000 is a wide host range strain originally isolated from tomato in French Guyana that belongs to the phylotype I (race 1, biovar 3). Draft genomes are available for three additional *R. solanacearum* strains belonging to phylotype II and exhibiting a narrow host range: IPO1609/UW551 (phylotype IIB, sequevar 1), which cause potato brown rot disease in cool-temperate climates and Molk2 (phylotype IIB, sequevar 3), which causes Moko disease of banana and plantain (Gabriel et al., 2006; Guidot et al., 2009b). Recently, the genome sequence of three additional broad host range strains from others phylotypes was realized: American strain CFBP2957 (phylotype IIA), African strain CMR15 (phylotype III) and Indonesian strain PSI07 (phylotype IV) (Remenant et al., 2010).

Genome sequencing has led to the development of high-throughput approaches to defining essentiality of genes on the genomic scale, gathering a large amount of information on plant-microbe interactions. With the availability of genome sequences of distinct *R. solanacearum* strains, new pathogenicity determinants have been identified by systematic screenings using different approaches like, in vivo expression technology (IVET), microarrays transcriptome analysis, transposon insertion mutagenesis and bioinformatics genomic comparisons (Brown and Allen, 2004; Occhialini et al, 2005; Gabriel et al., 2006; Lin et al., 2008). Comparative genomics hybridization (CGH) using microarray technology provided another alternative to explore on a genome-wide scale, the genetic diversity within any particular group of bacteria and to identify virulence factors and genes involved in environmental persistence of pathogens. The goal is to correlate those differences to biological functions and to gain insight into selective evolutionary pressures and patterns of gene transfers or losses, particularly within the context of virulence in pathogenic species. A pan-genome microarray of *R. solanacearum* covering the gene repertoire of three strins (GMI1000, IPO1609 and Molk2), was developed and applied in a CGH approach revealing new insights into the evolution and taxonomy of the *R. solanacearum* species complex (Guidot et al., 2007; Remenant et al., 2010).

In the present work, we used this approach to identify candidate genes involved in the interaction of *R. solanacearum* with potato as a host. As a starting point we rely on the existence of significant differences in aggressiveness among a collection of *R. solanacearum* strains isolated from potato fields in Uruguay. Based on this finding, we conducted a CGH analysis in order to establish the gene repertoire of each strain and allow us to perform comparisons between strains with differences in aggressiveness or source of isolation.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Tables 3-1 and 3-2. *R. solanacearum* strains were routinely cultured at 28ºC either in casamino acid peptone glucose (CPG) (Hendrick and Sequiera, 1984) or tetrazolium chloride agar (TZC) (Kelman, 1954). *Escherichia coli* strain DH5α used for genetic constructions was routinely cultured at 37ºC in Luria-Bertani (LB) medium (Sambrook et al., 2001). Antibiotics were added as required in the following final concentrations: ampicillin, 100 mg/l; spectinomycin, 40 mg/l; tetracycline, 10 mg/l.

Table 3-1. *Ralstonia solanacearum* wild-type strains used in this study

Strain	Origin			Phylotype-Sequevar	Aggressiveness[b]	Analysis
	Year	Location[a]	Sample			
Uruguayan strains						
UY031	2003	San José	Potato	IIB-1	High	CGH, PCR
UY032	2003	San José	Potato	IIB-1	High	CGH, PCR
UY033	2003	Tacuarembó	Potato	IIB-1	Low	CGH, PCR
UY034	2003	Tacuarembó	Potato	IIB-1	High	CGH, PCR
UY035	2003	Tacuarembó	Potato	IIB-1	Low	CGH, PCR
UY036	2003	Tacuarembó	Potato	IIB-1	High	CGH, PCR
UY041	2004	Rocha	Potato	IIB-1	High	CGH, PCR
UY042	2004	Rocha	Potato	IIB-1	High	CGH, PCR
UY043	2004	Tacuarembó	Soil	IIB-1	Low	CGH, PCR
UY082	2008	San José	Potato	IIB-1	High	PCR
UY084	2008	San José	Potato	IIB-1	High	PCR
UY087	2008	Rocha	Potato	IIB-1	High	PCR
UY091	2009	San José	Potato	IIB-1	High	PCR
Reference strains						
IPO1609	1995	Netherlands	Potato	IIB-1	Medium	CGH, PCR
GMI1000	1978	French Guyana	Tomato	I-18	ND	CGH, PCR

CGH: comparative genomic hybridization, PCR: polymerase chain reaction, ND: not determined

[a] Strains collected from all three potato-growing areas in Uruguay: San José (South), Tacuarembó (North) and Rocha (East). Strains belonged to different fields with the exception of strains UY031 and UY032 that were isolated from different tubers collected in the same field.

[b] Agressiveness level on tomato (cv. Loica), potato (cv. Chieftain) and the wild potato relative *S. commersonii*, based on the comparative aggressiveness analysis described in Chapter 2.

The growth of *R. solanacearum* wild-type and mutant strains was compared in liquid minimal medium (MM) (Bertolla et al., 1997) and in CPG medium. Cultures were inoculated in triplicate to and initial optical density of 0.02 and bacterial growth was monitored by measuring changes in optical density at 600 nm over time (Tarns-Kersten et al., 2001).

Table 3-2. *Ralstonia solanacearum* mutant strains, plasmids and primers used for genetic constructions

Strain, plasmid or primer	Relevant characteristics	Reference or source
R. solanacearum mutant strains		
UY031M2	Recombinant of strain UY031 by pGRUΩD, Sp^r	This study
GMI1750	GMI1000 derivative, *pil*A^-, Tc^r	C. Boucher[a]
Plasmids		
pGEM-T easy	Cloning vector, Ap^r	Promega
pGR-Ω	Sp^r, Ap^r	C. Boucher[a]
pGRU	PCR amplified (1300-bp) RSIPO_04290 upstream fragment, cloned in pGEM-T easy, Ap^r	This study
pGRD	PCR amplified (1450-bp) RSIPO_04295 downstream fragment, cloned in pGEM-T easy, Ap^r	This study
pGRUD	1450-bp insert from pGRD cloned into *Xba*I – *Sac*I sites of pGRU, Ap^r	This study
pGRUΩD	2000-bp Ω cassette from pGR-Ω inserted into *BamH*I sites of pGRUD, Sp^r, Ap^r	This study
Primers[b]		
UF4290	5'-TCTAGATGAACATGAACGAGGTGCTC-3' (*Xba*I)	This study
UR4290	5'-GGATCCTCAATCAGTTGGGTGTGC-3' (*BamH*I)	This study
DF4295	5'-GGATCCAGCGAACGGTATACGGAATG-3' (*BamH*I)	This study
DR4295	5'-GAGCTCCGACCTACCTGAACTGGAA-3' (*Sac*I)	This study

Ap^r, Sp^r, Tc^r: resistant to ampicillin (Ap), spectinomycin (Sp) or tetracycline (Tc)

[a] Strains or plasmids kindly provided by C. Boucher from INRA-CNRS, Toulouse, France.

[b] The underlined sequences correspond to restriction sites incorporated in primers corresponding to the enzymes specified in parenthesis

Identification of candidate pathogenicity genes

The repertoire of genes present in 9 *R. solanacearum* strains isolated from potato fields in Uruguay in 2003 and 2004 was established from comparative genomic hybridization (CGH) experiments using a microarray chip generated by C. Boucher and collaborators (INRA-CNRS, Toulouse, France), as described in Chapter 2. This spotted

microarray consisted of 6515 oligonucleotides representative of the genes identified from the genomes of three *R. solanacearum* strains belonging to different phylotypes or sequevars: GMI1000 (I-18), IPO1609 (IIB-1), Molk2 (IIB-3). Lists of candidate pathogenicity genes were generated by selecting specific genes for strains with different level of aggressiveness or source of isolation. From candidate genes referred in the microarray with the designation of strain GMI1000, the corresponding homolog in strain IPO1609 was searched using Basic Local Alignment *Search* Tool (*BLAST*) available in the *R. solanacearum* genome database (http://sequence.toulouse.inra.fr/R.solanacearum).

Validation of the differences observed in gene repertoires was performed by PCR amplification with specific primers designed for each gene based on the genome sequence of strain IPO1609, since it belongs to the same group as the Uruguayan strains (IIB-1). For candidate genes with no homolog in IPO1609 (Blast-P identity < 80%), primers were designed based on the sequence of strain GMI1000. Amplification was performed over 11 *R. solanacearum* strains with high aggressiveness (6 of which were also used for CGH experiments) and 3 strains with low aggressiveness (all used for CGH experiments) (Table 3-1). PCR reactions were conducted in a total volume of 25 µl containing 1× DNA polymerase buffer (Invitrogen), 1.5 mM $MgCl_2$, 10 pmol of each forward and reverse primers, 0.2 mM each of four dNTPs, 1 U of *Taq* DNA polymerase (Invitrogen) and 100 ng of genomic DNA. Amplification conditions included an initial denaturation step at 96°C for 5 min; followed by 35 cycles of 94°C for 20 s, 59°C for 30 s, and 72°C for 30 s; with a final extension step of 72°C for 10 min. Negative (PCR reaction mixture without DNA) and positive controls (IPO1609, or GMI1000 DNA) were included in each experiment. PCR reactions using universal primers for *R. solanacearum* 759/760 (Opina et al., 1997) with each tested DNA was conducted under the same conditions as a positive control of amplification. PCR products (8 µl) were analyzed by electrophoresis through 1.2 % (w/v) agarose gels with 0.5 µg/ml ethidium bromide and photographed under UV light.

General DNA manipulation and sequence analysis

Molecular cloning procedures including preparation of competent cells, ligation, transformation of *E. coli* by electroporation, genomic DNA extraction and DNA restriction and analysis, were performed according to standard protocols (Ausubel, et al., 1994; Sambrook et al., 2001). Restriction enzymes, DNA ligase, and other DNA enzymes were used according to the manufacturers' recommendations. Plasmid DNA was isolated using Wizard Plus SV Minipreps DNA Purification System (Promega). DNA sequencing and oligonucleotide synthesis were performed by Macrogen services (Kumchun-ku, Seoul, Korea). Vector NTI Advance 11.0 sequence analysis software package (Invitrogen), NCBI (www.ncbi.nlm.nih.gov/), and the genome database of *R. solanacearum* sequenced strains (http://sequence.toulouse.inra.fr/R.solanacearum) and http://vision.biotech.ufl.edu/mycap) were used to view and analyze DNA sequences.

Construction of *R. solanacearum* mutant strains

A *R. solanacearum* mutant strain was generated by inserting the Ω interposon carrying a spectinomycin resistance gene (Prentki and Krisch, 1984) in substitution of the gene cluster comprised between genes RSIPO_04290 and RSIPO_04295 in the high aggressive strain UY031. Primers were designed based on strain IPO1609 genome draft in order to amplify a 1300-bp fragment located upstream the gen RSIPO_04290 (primer pair UF4290-UR4290) and a 1450-bp fragment downstream the gene RSIPO_04295 (primer pair DF4295-DR4295) (Table 3-2). Specific restriction sites were incorporated to each primer for used in subsequent cloning steps. The PCRs were performed in 25-µl reaction mixtures containing 1× Phusion GC buffer (with $MgCl_2$), 1.25 U of Phusion DNA polymerase (New England Biolabs, Inc.), dNTPs (0.2 mM each), forward and reverse primers (0.4 µM each), and 50 ng of genomic DNA from strain UY031 as template. The thermocycling conditions were 98°C for 30 s; 35 cycles of 98°C for 10 s, 60°C for 30 s, and 72°C for 45 s; and a final extension step at 72°C for 5 min. The resulting fragments were cloned into pGEM-T easy (Promega), creating pGRU and pGRD, and the inserts were confirmed by sequencing with vector primers SP6 and T7. The downstream fragment was excised from pGRD by double digestion with *BamH*I

and *Sac*I, and inserted into pGRU to produce pGRUD. The Ω-spectinomycin resistance cassette was introduced into pGRUD, at a unique *BamH*I site located between upstream and downstream fragments, generating pGRUΩD. In order to verify this construction, multiple digestions were performed (*BamH*I, *BamH*I/*Sac*I, and *BamH*I/*Sca*I) and analyzed by agarose gel electrophoresis. Finally, this construction was introduced into the genome of wild-type *R. solanacearum* strain UY031 by natural transformation and homologous double recombination following the protocol described by Boucher et al. (1985) with minor modifications. Briefly, *R. solanacearum* strain UY031 was grown at 28°C in minimal medium MM to an optical density at 600 nm ranging from 0.5 to 1.0. Thirty microliters of this cell suspension was directly mixed with 100 ng (3 µl) of plasmid preparation (pGRUΩD) in nitrocellulose membranes deposited on the surface of solid CPG medium, and incubated for 48 h at 28°C. Bacterial cells were then harvested from the membrane surface, resuspended in 1 ml of sterile water and 100 µl aliquots were plated in TZC medium with spectinomycin. Candidate colonies appeared after incubation at 28ºC for 2 to 5 days and were further verified by extracting genomic DNA and performing PCR amplification with primers for each deleted gene (Table 2-3). A resistant mutant strain (UY031M2) with typical colony morphology in TZC medium was selected and used for phenotypic characterization.

Twitching motility assay

To observe twitching motility, plates were prepared and incubated as described by Liu et al. (2001) with minor modifications. Briefly, freshly poured CPG plates were dried by removing their lids and placing them inverted in a 37ºC incubator for 10-30 min. Inoculation was performed by spotting microdroplets (10 µl) of cell suspensions adjusted to 10^5cfu/ml on the surface of the plate. Plates were incubated at 28ºC in a loosely closed plastic bag along with several moist paper towels to ensure high humidity. After 24 – 30 h colonies were examined for twitching motility by placing a Petri dish without its lid on the stage of an upright light microscope (Olimpus BX41) using the 10x objective.

Plant assays

The virulence of wild-type strains (UY031 and UY043) and mutant strain UY031M2 was measured on susceptible *Solanum tuberosum* cv. Chieftain, using both a direct petiole inoculation and a soil inoculation procedure. Plantlets were micro-propagated from single-node pieces growing in vitro on Murashige and Skoog (MS) medium supplemented with 30 g of sucrose per liter and maintained at 22ºC and 16 h photoperiod. After three weeks, plantlets were transferred into plastic flats with 17 cm^3 cells containing commercial soil mix (TREF, Moerdijk, The Netherlands), and placed for acclimatization in a greenhouse at 22 to 25ºC under natural light for two weeks prior to inoculation. To prepare the inocula, strains were grown overnight at 28ºC and agitation at 200 rpm in liquid CPG or CPG-spectinomycin, for wild-type and mutant strains respectively. Cells were pelleted by centrifugation, suspended in 0.9 % saline solution and spectrophotometrically adjusted to 10^8cfu/ml (optical density at 600 nm of 0.1). Inocula concentrations were checked by dilution plating on TZC or TZC-spectinomycin agar to observe typical smooth colonies after 2-day incubation at 28ºC. These suspensions were used directly or diluted in sterile saline solution for plant inoculations. For direct petiole inoculation, the third true leaf from the top of the plant was excised 5 mm from its base, and a 10-µl droplet of a bacterial suspension diluted to 10^5cfu/ml was placed onto the cut surface (equivalent to 1000 cells). Five replicates plants were inoculated with each strain in a completely randomized design and the experiment was repeated twice, giving a total of 10 tested plants for each strain.

Soil inoculation was performed following a procedure adapted from Montanelli et al. (1995). Prior to inoculation, roots were slightly damaged by making a hole with a 1000 µl disposable pipette tip next to each plant, and then one ml of the bacterial suspension adjusted to 10^8cfu/ml was poured inside the hole (5 x 10^6 cfu/g soil). A set of 10 potato plants were inoculated with each strain and placed in separated trays in order to prevent cross contamination. Three replicate trays were assayed for each strain in a completely randomized design and the experiment was repeated twice under the same conditions.

Plants inoculated with saline solution were used as negative controls in all experiments. After inoculation, plants were incubated in a growth chamber under

28ºC with 16 h photoperiod. Disease development was recorded at regular time intervals using an ordinal scale ranging from 0 (no wilting symptoms) to 4 (all leaves wilted) up to 21 days after inoculation (Winstead and Kelman, 1952). Strain aggressiveness was estimated by the area under disease progression curve (AUDPC) based on average wilt scoring for each strain and inoculation method. AUDPC data was subjected to analysis of variance (ANOVA) and means were compared using Tukey's multiple comparison procedure using INFOSTAT software (Di Rienzo et al., 2009)

Colonization assays

To study colonization fitness of wild-type and mutant *R. solanacearum* strains, bacterial populations, in *S. tuberosum* cv. Chieftain plants, were measured after an in vitro root inoculation assay adapted from Vailleau et al. (2007). Experiments were performed with plantlets growing in axenic conditions in nutrient MS medium supplemented with 30 g of sucrose and 0.1 mg of indole-3-butyric acid per liter. Potato plantlets were grown from single-node pieces in square petri dishes (12 cm x 12 cm), with solid nutrient medium covered with filter paper in order to force the roots to growth on the surface. Petri dishes were sealed with Parafilm with several incisions to allow gas exchange, and maintained inclined with an angle of 45º at 22ºC and 16 h photoperiod. After two weeks, plantlets were inoculated on intact root tips by applying 0.3 ml of a bacterial suspension adjusted to 10^7cfu/ml along the entire length of roots. Petri dishes containing 6 plantlets were inoculated with each tested strain in a completely randomized design and held inclined in a growth chamber under 28ºC and 16 h photoperiod. Negative controls were carried out using the same procedure with saline solution. After 1, 4 and 7 days, three replicate Petri dishes inoculated with each strain were harvested, and the plantlets were pooled and processed for internal bacterial density counts. Root systems and stem segments (approximately 3 cm in length) were removed from each plant and assayed separately. Plant tissues were surface sterilized by immersion in 1.0 % sodium hypochlorite solution for 1 min, washed three times with sterile water, and dried with absorbent paper. Each sample was weighted and ground in 5 ml of distilled water by use of sand with a mortar and pestle. The resulting root and stem homogenates were subjected to 10-fold serial

dilutions, plated onto TZC or TZC-spectynomicin agar medium, and colonies were counted after 2 days of incubation at 28°C. *R*. *solanacearum* population densities were normalized to log (cfu/g) of plant tissue and were analyzed by using ANOVA. The Tukey's test was applied to compare the differences among the treatments at the 95% confidence level. All statistical analysis was performed by using Infostat software (Di Rienzo et al., 2009).

RESULTS

Identification and validation of candidate pathogenicity genes in *R. solanacearum*

In this study, we used a subset of the CGH-microarray data generated in Chapter 2 for 9 *R. solanacearum* strains isolated from potato fields in Uruguay in 2003 and 2004 (Table 3-1). These strains were previously characterized in a comparative aggressiveness analysis on various hosts, revealing different aggressiveness levels (see Chapter 2). Based on the gene content of each strain established by CGH experiments, lists of candidate pathogenicity genes were generated by selecting specific genes for strains with different level of aggressiveness or source of isolation (Table 3-3). To support the microarray evidence, we performed the PCR amplification of one genomic fragment for each candidate gene. First, all tested genomic DNAs were amplified with universal primers for *R. solanacearum* (Opina et al., 1997) yielding a product of the expected size (282-bp) (Figure 3-1). Therefore, we confirmed that all tested strains actually correspond to *R. solanacearum* isolates, and that these DNA samples could be used for the PCR-validation of the candidate pathogenicity genes.

In a first attempt, 9 genes were identified as present in all 6 strains with high aggressiveness and absent in all 3 strains with low level of aggressiveness (Table 3-3A). However, none of these genes were validated by PCR (Figure 3-1).

A second genomic comparison was performed in order to identify specific differences in gene content of strain UY043. This strain displayed low aggressiveness on several hosts and was isolated from soil, while the others *R. solanacearum* strains were isolated from potato tubers with typical bacterial wilt symptoms. Therefore, we focused the analysis to identify any genomic changes incurred in a strain from the open environment, which may explain their low level of aggressiveness (Table 3-3B and 3-3C). By comparison with the strain IPO1609 draft genome, it was noticed that 25 genes identified as absent in strain UY043 were found to localize to one single genomic region of 33.7-kb in size. Validation by PCR amplification confirmed that all the 25 genes that comprise this region were absent in strain UY043 and present in 12 *R. solanacearum* strains from Uruguay, as well as in reference strain IPO1609 (Figure 3-2). In addition, 5 isolated candidate genes were identified, but only 2 (RSIPO_03704 and

RSIPO_03609) were validated by PCR, since for some genes an amplification product of the expected size was obtained even for strain UY043. Finally, 2 genes were identified and PCR validated as exclusively present in strain UY043 (Figure 3-3).

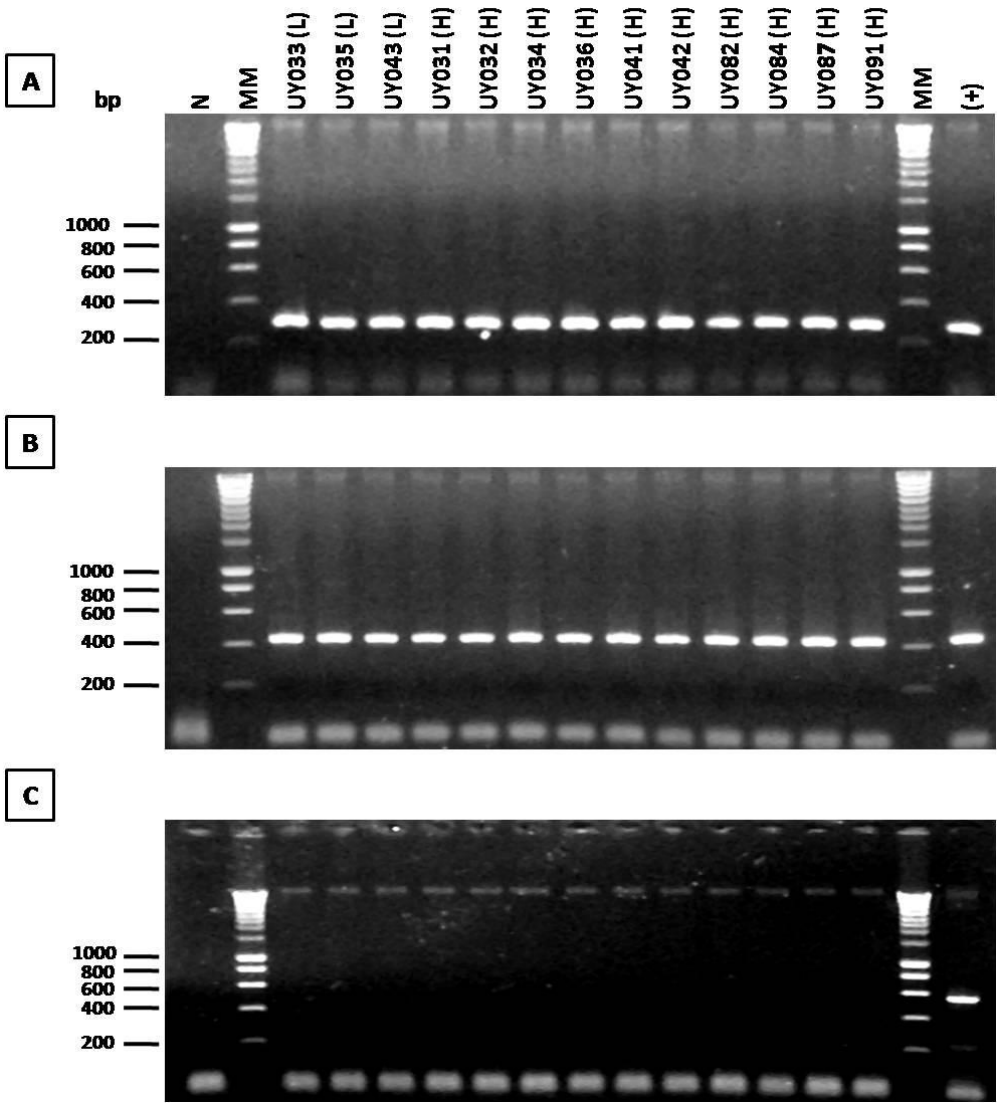


Figure 3-1. Representative results obtained for the amplification of candidate pathogenicity genes identified in Table 3-3A using genomic DNA from 13 *Ralstonia solanacearum* strains from Uruguay with high (H) and low (L) aggressiveness, a positive control (+) of amplification (strain IPO1609 for A, B, and strain GMI1000 for C), and water as a negative control (N). A: PCR amplification of a 282-bp fragment with universal primers for *R. solanacearum* 759-760 (Opina et al., 1997). B: PCR amplification of a 424-bp fragment of RSIPO_03403 gene. C: PCR amplification of a 552-bp fragment RSc0624 gene. MM = Molecular weight marker (Smart Ladder, Eurogentec, 10-kb).

Table 3-3. Lists of candidate pathogenicity genes identified through a microarray comparative genomic analysis of Uruguayan *Ralstonia solanacearum* strains with different aggressiveness levels and source of isolation. Grey boxes are used to indicate genes that were validated by PCR amplification using specific forward (F) and reverse (R) primers design for each candidate gene.

A. Genes present in strains UY031, UY032, UY034, UY036, UY041, and UY042 and absent in strains UY033, UY035 and UY043					
Gene ID [a] (GMI1000)	**Gene ID [a] (IPO1609)**	**Predicted function [a]**	**Primer F [b]**	**Primer R [b]**	**PCR product size (bp)**
RSc0624	NH	Zinc-dependent alcohol dehydrogenase oxidoreductase protein	CTCGTTGCTTACGCTGTCTG	TAGCCATCTGGGACTTCGTC	552
RSc0853	NH	Bacteriophage-related protein	ACCTGTTCCTCGATGAGGTG	TGTACAGCGAGGACAAGTGG	401
RSc0858	NH	Bacteriophage-related protein	CCCAAACTTGACGTGATCCT	GTGTGGGTTGAGGTCGTTCT	543
RSc1635	RSIPO_01191	t-rna-dihydrouridine synthase c protein (dusC)	GCTTCTACGAACGGGAAACC	CAGCGAGGTATCGGAGACAC	317
RSc2129	NH	Putative D-arabinitol dehydrogenase oxidoreductase protein (dalD)	AATGGGTGATCGAGGACAAC	GACGTAGTCGTAGGCGAAGC	218
RSc2536	RSIPO_04117	Transmembrane aldehyde dehydrogenase protein	CCAAGGTCGAAGGCTACATC	GCTGCATCACGCTCTTGTAG	450
RSp0201	RSIPO_04285	Conserved hypothetical protein	CTGAAAGTGGTGCAGGTCAA	GGTAGTCCTCCTGCTCGATG	277
RSp0900	RSIPO_03403	Secreted protein popF2	GACTCGAACGCCAAGAAGAC	TGCTGGACATGAACTTCTCG	424
RSp1473	NH	Putative oxidoreductase protein	ACCAGGGCATCAACTTCATC	CGGTCGACGAGGTTGTAGAG	427
B. Genes present in strains UY031, UY032, UY033, UY034, UY035, UY036, UY041, and UY042 and absent in strain UY043					
Gene ID [a] (GMI1000)	**Gene ID [a] (IPO1609)**	**Predicted function [a]**	**Primer F [b]**	**Primer R [b]**	**PCR product size (bp)**
RSp1277	RSIPO_04287 [c]	Putative type III effector protein	ATGTGAAGCAGCAACTCACG	GTCTCGGTCAGGAGATCGAC	544
RSp1278	RSIPO_04288 [c]	Multidrug transporter protein	TTCGTCTCCCTCGACAAATC	CGACGGAAAAGAGGAAGATG	262
RSp1279	RSIPO_04289 [c]	Fumarate hydratase (*fumC*)	TGAACATGAACGAGGTGCTC	CAGTGCCAGTTCACACAGGT	371
RSp1287	RSIPO_04290 [c]	Tfp biogenesis protein (*pilE1*)	GCACACCCAACTGATTGATG	CGATTGCCTATCCGTCCTAC	333

Table 3-3B. Continue

Gene ID [a] (GMI1000)	Gene ID [a] (IPO1609)	Predicted function [a]	Primer F [b]	Primer R [b]	PCR product size (bp)
RSp1288	RSIPO_04291 [c]	Tfp biogenesis PilY1-related protein	CGTAATCGGTGATGTGATCG	CATCGTATTCGCACAACCTG	540
RSp1289	RSIPO_04292 [c]	Putative transmembrane Tfp assembly protein (*pilX*)	CAATCTGATACGTGCTCTGC	CCGTTTCAATCGTCATCTCC	550
RSp1290	RSIPO_04293 [c]	Putative Tfp biogenesis transmembrane protein (*pilW*)	CTGATGTTCTGGACCCACTG	TCATCTCGCTGGTGATCTTG	646
RSp1291	RSIPO_04294 [c]	Putative Tfp biogenesis PILV transmembrane protein (*pilV*)	GCTGTAGCTCGGAAACTTCG	TCAGCAGTACGCAGAACTCG	269
RSp1292	RSIPO_04295 [c]	Putative Tfp pilin related pilin	TCACGGTTGTAGGTCAGCAC	ACAGCAGCAGCAACAACAAG	172
RSp1293	RSIPO_04296 [c]	S-adenosylmethionine decarboxylase related protein	GAACACCTGCTGCTCGATCT	GGATGGATAGATGGGATTCG	217
RSp1294	RSIPO_04297 [c]	Serin-rich transmembrane protein			
RSp1295	RSIPO_04298 [c]	Hypothetical transmembrane protein	CTCAAGGACATCGGCAAGAT	GTAGTCGAGCGTCAGGAAGG	423
RSp1296	RSIPO_04299 [c]	Hypothetical transmembrane protein	GGACATGGAAATCCAGAACG	GGTAGAACAGCTTGGGATCG	335
RSp1297	RSIPO_04300 [c]	Hypothetical transmembrane protein	TACCTCAAGCTGACGCCGTA	CGTGTTGTTGTGCTCGATCT	246
RSp1298	RSIPO_04301 [c]	ABC-type branched-chain amino acid transport system protein	GGTCAGCAACGAGAAGAAGG	TGATCAGGTAGGCGGAGAAG	302
NH	RSIPO_04302 [c]	Amino acid transport protein	CTGTCGCTGATCCTGAACCT	GGTAGCCGGTGATGAAGAAG	346
NH	RSIPO_04303 [c]	Amino acid transport protein	CGCCGATGCGTTCTATTACT	CAGGCTCGACAGGTAGTCCT	367
NH	RSIPO_04304 [c]	Amino acid transport protein	GTGCAAGAGGTCTCGCTGTC	GATCTCGGTGATCTGGAACG	213
NH	RSIPO_04305 [c]	Amino acid transport protein	ACTACGGCAAGAGCCACATC	CGGGAACAGTTCGTAGATGC	323
RSp1337	RSIPO_04306 [c]	Probable spermidine synthase 2 protein (*speE2*)	ACGAGTACCGCTACCACGAG	GAATACAGCTTGCCGATGCT	346
RSp1307 RSp1338	RSIPO_04307 [c]	Conserved hypothetical protein	ACTGGTACGCCGACTACTGC	CGGTGAAGACGGACTGCT	561

Table 3-3B. Continue

Gene ID [a] (GMI1000)	Gene ID [a] (IPO1609)	Predicted function [a]	Primer F [b]	Primer R [b]	PCR product size (bp)
RSp1308 RSp1339	RSIPO_04308 [c]	Putative dienelactone hydrolase protein	ACCTGATGACCCACCTGAAC	ATCTTGAAGAAGCCGTCCTG	265
RSp1340	RSIPO_04309 [c]	Conserved hypothetical protein	CCCCTATACCGCCTTCAACT	CGACCGTCTCAAGACACAGA	352
RSp1374	RSIPO_04310 [c]	Type III effector protein SKWP2	GTCTGGTCCACCGAAGACAT	TGCAGCAAATCTTCCAGTTG	563
RSp1379	RSIPO_04311 [c]	Probable signal peptide protein	AGCGAAGTCAAATGGGAATG	GACCAGCCGAGCACATAGAT	320
RSp0522	RSIPO_03704	Hypothetical protein	CGGTGGTGTACTTGGAGGTT	ACGTCCTGGTACTGCCTGTC	556
RSp0888	NH	Oxidoreductase protein	GGTTCTGGACGGAGAAACTC	GCCGGTGGAGTATTCGTAGA	290
RSp0970	RSIPO_03609	Proteasa protein	TTACCTGGGCCTGAAGAATG	ACTGTTCACGCATCAGTTCG	326
RSp1084	RSIPO_04154	Pilus assembly transmembrane protein	TCTGTACGTCCTGGTGTTCG	AGCAGGACCAGTTCGTTGAC	369
RSp1141	NH	Transcription regulator protein	TCATCCTGCTGGACTTTGC	TGCTGCATATGGCTCTTCAC	295
C. Genes present in strain UY043 and absent in strains UY031, UY032, UY033, UY034, UY035, UY036, UY041, and UY042					
Gene ID [a] (GMI1000)	**Gene ID [a] (IPO1609)**	**Predicted function [a]**	**Primer F [b]**	**Primer R [b]**	**PCR product size (bp)**
RSp0139	NH	GCN5-related N-acetyltransferase	CAGATCACCGAAGACGTTCA	CAGCAGCGTCTCGATCAAC	261
RSp0326	RSIPO_04459	Organic hydroperoxide resistance protein	AGGTCTCGCATGACTTGTCC	CGTTGGAATACGGACAGACC	389

[a] Gene designation and description based on annotation for *R. solanacearum* strains GMI1000 and IPO1609.

[b] Primers used in PCR validation of candidate genes were designed based on the genome sequences of strain IPO1609, except for those genes with no homolog (NH) in strain IPO1609 (Blast-P identity < 80%). .

[c] Genes comprised in a 33.7-kb region absent in strain UY043 (DR043) identified by comparison with genome sequence of strain IPO1609.

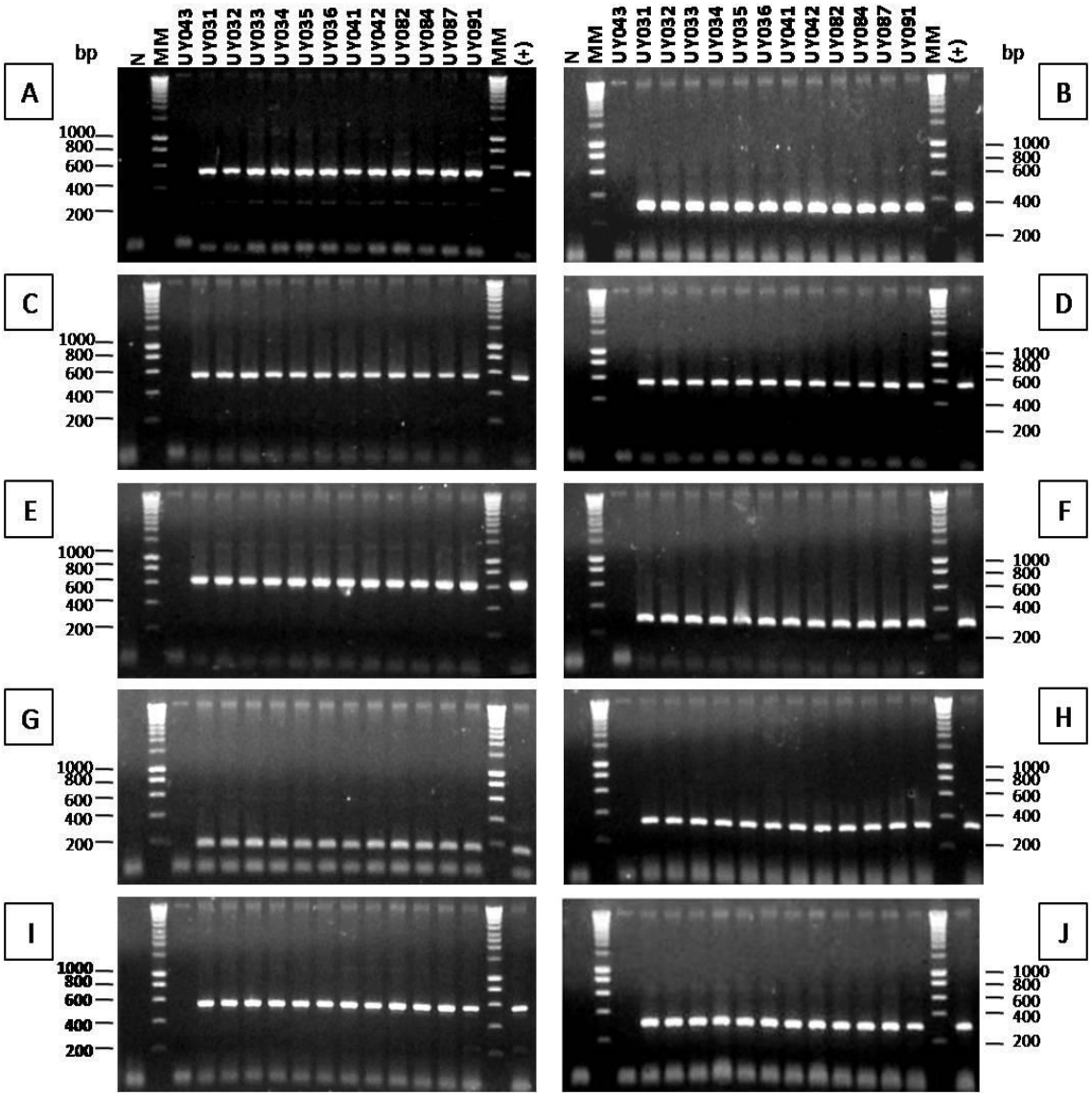


Figure 3-2. Representative results for the validation of candidate genes absent in strain UY043 and present in others *Rasltonia solanacearum* strains from Uruguay (Table 3-3B). This validation was conducted by polymerase chain reaction (PCR) amplification of one genomic fragment from each candidate gene using genomic DNA from 13 *R. solanacearum* strains from Uruguay, the IPO1609 strain as a positive control (+), and water as a negative control (N). A: Amplification of a 544-bp fragment of RSIPO_04287. B: Amplification of a 333-bp fragment of RSIPO_04290. C: Amplification of a 540-bp fragment of RSIPO_04291. D: Amplification of a 550-bp fragment of RSIPO_04292. E: Amplification of a 646-bp fragment of RSIPO_04293. F: Amplification of a 269-bp fragment of RSIPO_04294 gene. G: Amplification of a 172-bp fragment of RSIPO_04295. H: Amplification of a 346-bp fragment of RSIPO_4302 gene. I: Amplification of a 563-bp fragment of RSIPO_04310. J: Amplification of a 326-bp fragment of RSIPO_03609. MM = Molecular weight marker (Smart Ladder, Eurogentec, 10-kb).

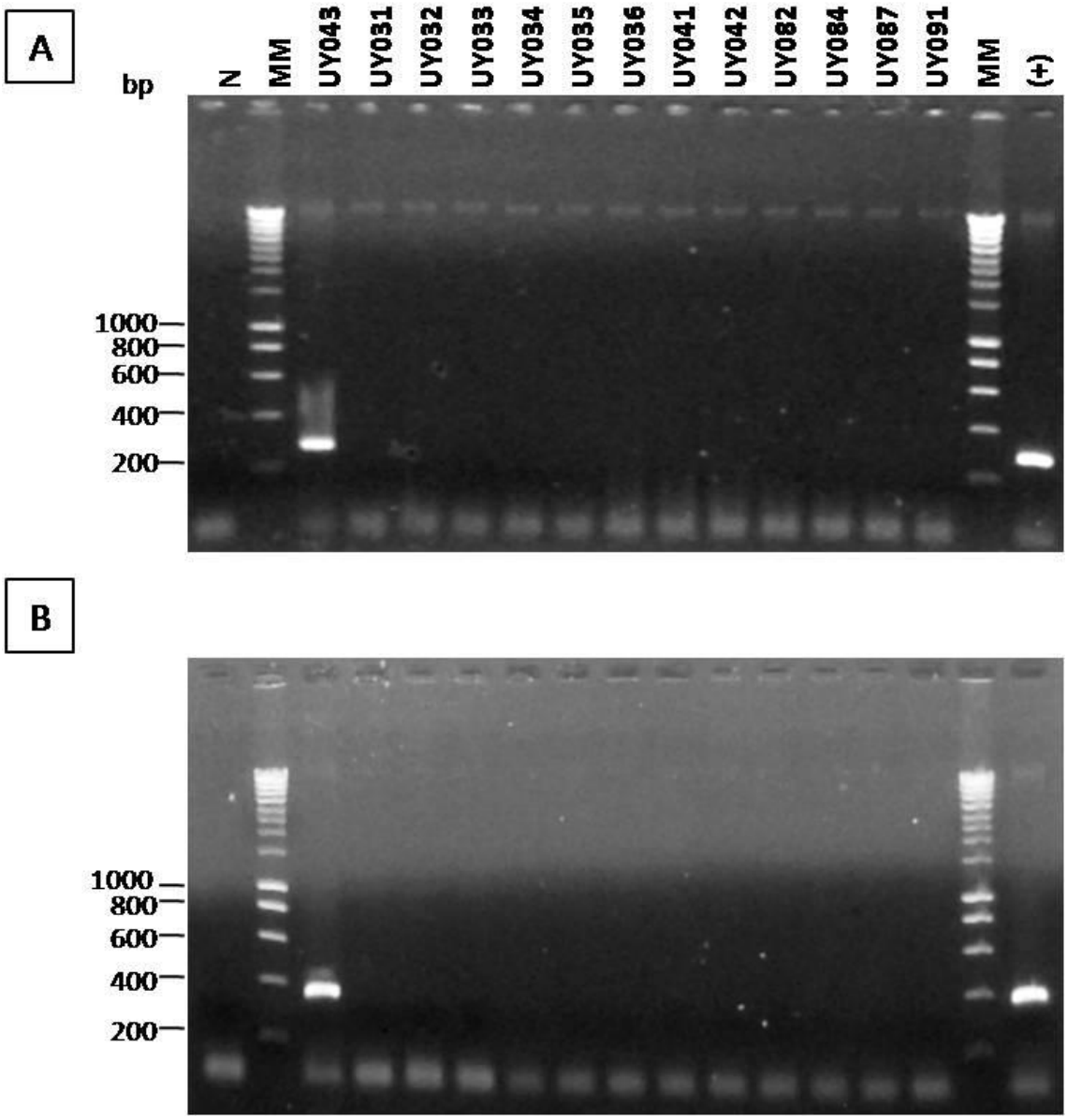


Figure 3-3. Validation of genes RSp0139 (A) and RSIPO_04459 (B) as present in strain UY043 and absent in other *Ralstonia solanacearum* strains from Uruguay. This validation was conducted by polymerase chain reaction (PCR) amplification of one genomic fragment from each candidate gene using primers described in Table 3-3C and genomic DNA from 13 *R. solanacearum* strains from Uruguay, the IPO1609 strain as a positive control (+), and water as a negative control (N). MM = Molecular weight marker (Smart Ladder, Eurogentec, 10-kb).

Description of the deleted region in strain UY043 (DR043)

Since most of the identified candidate genes share the same location within the genome, we decided to concentrate our research on this region denoted as DR043. Based on information from strain IPO1609 draft genome, DR043 contained 25 genes covering 33.7-kb (Figure 3-4). The entire region possess an average G+C content of 66.7 %, equal to the average G+C content of the complete genome. Interestingly, the region is flanked by two partial transposase sequences showing a bias GC content, thus suggesting an external origin. To investigate a possible deletion mechanism, the sequences covering the borders of the region were compared, revealing a 480-bp sequence with high homology (98.3 %). Analysis of the strain UW551 draft genome database (Contig05764, position 42,531 – 77,542), revealed almost complete identity with strain IPO1609 with respect to the 25 genes present on DR043, although the length of genes that had been annotated was somewhat different (data not shown).

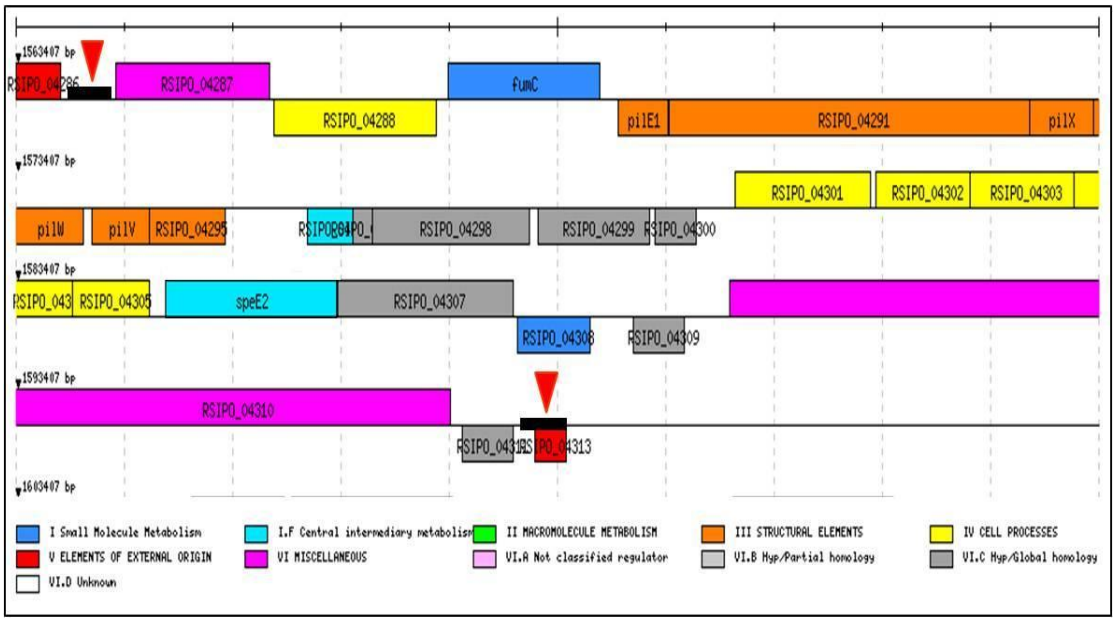


Figure 3-4. Genetic map of *Ralstonia solanacearum* strain IPO1609 corresponding to the identified deleted region in strain UY043 (DR043). The region of 33.7-kb contained 25 genes (RSIPO_04287 – RSIPO_04311) and is flanked by two partial transposase sequences (red boxes). Black boxes marked with a red arrow indicate a 480-bp sequence with high homology flanking the region.
Figure adapted from the *R. solanacearum* genome database from LIPM-INRA, Toulouse: http://sequence.toulouse.inra.fr/R.solanacearum

According to the annotation for strain IPO1609, the region contained 25 open reading frames (ORF) that putatively encode: (i) 2 proteins directed involved in virulence identified as protein effectors secreted through the type III secretion system (T3SS) (ORF1 and ORF24, RSIPO_04287 and RSIPO_04310), (ii) a cluster of 6 genes related to the biogenesis of type IV pili (ORFs 4-9, RSIPO_04290-RSIPO_04295), (iii) a cluster of 5 proteins involved in amino acid transport (ORFs 15-19, RSIPO_04301-RSIPO_4305), (iv) a multidrug transport protein (ORF2, RSIPO_04288), (v) 2 proteins involved in biosynthesis of spermidine (ORF 10 and ORF 20, RSIPO_04296 and RSIPO_4306), (vi) 2 proteins related to the metabolism of small molecules (ORF 3 and ORF 22, RSIPO_04289 and RSIPO_04308 10), and (vii) 7 proteins with unknown function.

To allow a cross-comparison of the identified genomic region among other *R. solanacearum* strains belonging to different phylotypes, we searched for the corresponding homologues proteins in 5 additional sequenced strains (Table 3-4 and Figure 3-5). This comparison led us to identify that the region DR043 is in fact located within the megaplasmid. Interestingly, high conservation was found between strains belonging to the phylotype II, since for Molk2 and CFBP2957 all 25 ORFs were clustered in the same order and shearing high homology with those of strain IPO1609. In strains belonging to other phylotypes, a greater degree of variation was observed and some genes were absent or additional genes were found interspersed between the ORFs identified in IPO1609. For instance, the corresponding region in strain GMI1000 showed a group of 8 conserved additional genes located between ORF3 and ORF4, and completely lacked ORFs 16 to 19 as well as ORF23, which also were not present elsewhere in the genome. Instead, this region in strain GMI1000 is interrupted by insertion sequence elements and transposons as well as a region with alternative codon usage (ACUR), suggesting the occurrence of a genome rearrangement within this region during evolution.

Table 3-4. Open reading frames (ORFs) present on DR043 in six *Ralstonia solanacearum* sequenced strains belonging to different phylotypes

ORF	Size [a] (aa)	Predicted function[b]	Nomenclature[c]					
			IPO1609 (IIB-1)	Molk2 (IIB-3)	CFBP2957 (IIA-36)	GMI1000 (I-18)	CMR15 (III-29)	PS107 (IV-10)
1	474	Putative type III effector protein	RSIPO_04287	RSMK03370	RCFBP_ mp30115	RSP1277	CMR15v4_mp20380	Absent
2	501	Multidrug transport protein	RSIPO_04288	RSMK03369	RCFBP_ mp30116	RsP1278	CMR15v4_mp20381	RPSI07_mp1317
3	469	Fumarate hidratase (*fumC*)	RSIPO_04289	RSMK03368	RCFBP_ mp30117	RSP1279	CMR15v4_mp20382	RPSI07_mp1318
4	155	Tfp biogenesis protein (*pilE1*)	RSIPO_04290	RSMK03367	RCFBP_ mp30118	RSP1287	CMR15v4_mp20390	RPSI07_mp1319
5	1111	Tfp biogenesis PilY1-related protein	RSIPO_04291	RSMK03366	RCFBP_ mp30119	RSp1288	CMR15v4_mp20391	RPSI07_mp1320
6	195	Putative transmembrane Tfp assembly protein (*pilX*)	RSIPO_04292	RSMK03365	RCFBP_ mp30121	RSp1289	CMR15v4_mp20392	RPSI07_mp1321
7	226	Putative Tfp biogenesis protein(*pilW*)	RSIPO_ 04293	RSMK03364	RCFBP_ mp30122	RSp1290	CMR15v4_mp20393	RPSI07_mp1322
8	180	Putative Tfp biogenesis protein (*pilV*)	RSIPO_ 04294	RSMK03363	RCFBP_ mp30123	RSp1291	CMR15v4_mp20394	RPSI07_mp1323
9	234	Putative Tfp related pilin	RSIPO_ 04295	RSMK03362	RCFBP_ mp30124	RSp1292	CMR15v4_mp20395	RPSI07_mp1324
10	140	S-adenosylmethionine decarboxylase protein	RSIPO_ 04296	RSMK03361	RCFBP_ mp30125	RSp1293	CMR15v4_mp20399	RPSI07_mp1328
11	61	Serin-rich transmembrane protein	RSIPO_ 04297	RSMK03360	RCFBP_ mp30126	RSp1294	CMR15v4_mp20400	RPSI07_mp1329
12	484	Hypothetical transmembrane protein	RSIPO_ 04298	RSMK03359	RCFBP_ mp30127	Absent	CMR15v4_mp20401	RPSI07_mp1330
13	342	Hypothetical transmembrane protein	RSIPO_ 04299	RSMK03358	RCFBP_ mp30128	RSp1296	CMR15v4_mp20402	RPSI07_mp1331
14	129	Hypothetical transmembrane protein	RSIPO_ 04300	RSMK03357	RCFBP_ mp30129	RSp1297	CMR15v4_mp20403	RPSI07_mp1332
15	419	Amino acid transport protein (Leu/Ile/Val-binding protein)	RSIPO_ 04301	RSMK03356	RCFBP_ mp30130	RSp1298	CMR15v4_mp20404	RPSI07_mp1337

Table 3-4. Continue

ORF	Size[a]	Predicted function[b]	Nomenclature[c]					
			IPO1609 (IIB-1)	**Molk2 (IIB-3)**	**CFBP2957 (IIA-36)**	**GMI1000 (I-18)**	**CMR15 (III-29)**	**PS107 (IV-10)**
16	289	Amino acid transport protein	RSIPO_ 04302	RSMK_03355	RCFBP_ mp30131	Absent	CMR15v4_mp20405	RPSI07_mp1338
17	322	Amino acid transport protein	RSIPO_ 04303	RSMK_03354	RCFBP_ mp30132	Absent	CMR15v4_mp20406	RPSI07_mp1339
18	249	Amino acid transport protein	RSIPO_ 04304	RSMK_03353	RCFBP_ mp30133	Absent	CMR15v4_mp20407	RPSI07_mp1340
19	236	Amino acid transport protein	RSIPO_ 04305	RSMK_03352	RCFBP_ mp30134	Absent	CMR15v4_mp20408	RPSI07_mp1341
20	527	Spermidine synthase 2 (*speE2*)	RSIPO_ 04306	RSMK_03351	RCFBP_ mp30135	RSp1337 RSp1306	CMR15v4_mp20409	RPSI07_mp1342
21	540	Conserved hypothetical protein	RSIPO_ 04307	RSMK_03348	RCFBP_ mp30136	RSp1338 RSp1307	CMR15v4_mp20410	RPSI07_mp1343
22	225	Putative dienelactone hydrolase protein	RSIPO_ 04308	RSMK_03346	RCFBP_ mp30137	RSp1338 RSp1308	CMR15v4_mp20411	RPSI07_mp1344
23	157	Conserved hypothetical protein	RSIPO_ 04309	RSMK_03344	RCFBP_ mp30139	Absent	Absent	Absent
24	2475	Type III effector protein (SKWP2)	RSIPO_ 04310	RSMK_03343 RSMK_03341	RCFBP_ mp30140	RSp1374	CMR15v4_mp30022	RPSI07_mp1356
25	157	Probable signal peptide protein	RSIPO_ 04311	RSMK_03340	RCFBP_ mp30141	RSp1379	CMR15v4_mp30037	RPSI07_mp1357

[a] Number of amino acids (aa) of each ORF according to *R. solanacearum* strain IPO1609 draft genome

[b] Gene function based on annotation for *R. solanacearum* strain IPO1609

[c] ORFs identified in *R. solanacearum* strains Molk2, CFBP2957, GMI1000, CMR15 and PS107 after Blast-P of the 25 ORFs of strain IPO1609 (Identity > 80%)

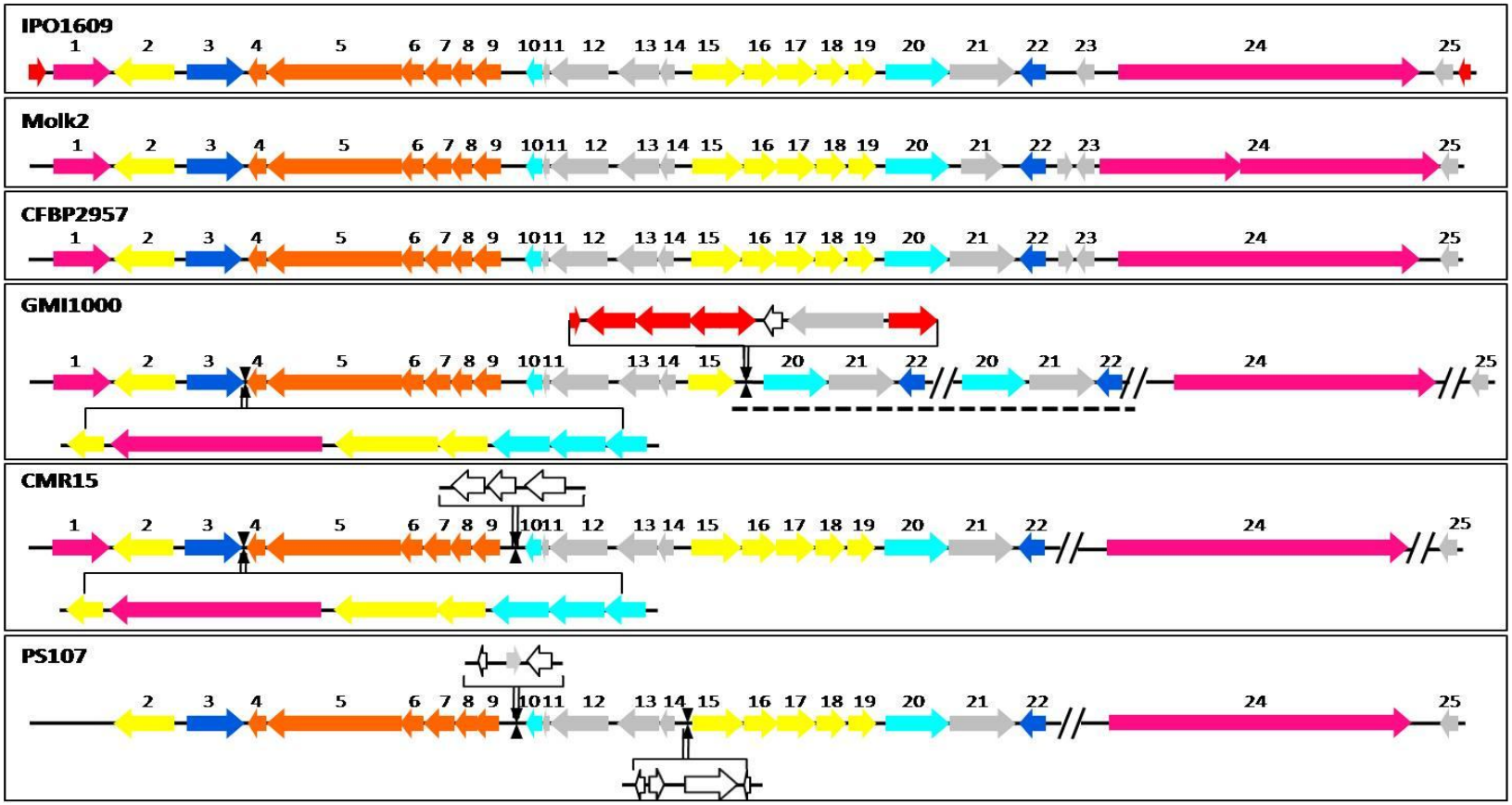


Figure 3-5. Comparison of region DR043 across *Ralstonia solanacearum* sequenced strains belonging to different phylotypes: IPO1609 (IIB-1), Molk2 (IIB-3), CFBP2957 (IIA-36), GMI100 (I-18), CMR15 (III-29), and PS107 (IV-10). Numbers above arrows indicate ORF number as in Table 3-4, and color code represents different gene classes according the *R. solanacearum* genome database from LIPM-INRA, Toulouse. ■: small molecule metabolism; ■: central intermediary metabolism; ■: structural elements; ■: cell processes; ■: elements of external origin; ■: miscellaneous (virulence); ■: hypothetical protein; □: unknown. For strains GMI1000, CMR15 and PS107, additional genes not present in strain IPO1609 were added above or below the central map (⧓). ▪▪▪▪▪▪▪▪▪ : Alternative codon usage regions (ACUR).

Function analysis of candidate genes

Analysis of annotation data for genes located in DR043 region, revealed several candidate genes that might be related with virulence traits. In this study, we focused the analysis on a genetic cluster involved in type IV pili (Tfp) biogenesis: genes RSIPO_04290 to RSIPO_04295. To assess whether the deletion of this cluster could explain the low aggressiveness of *R. solanacearum* strain UY043, a mutant strain was constructed by inserting a spectinomycin resistance cassette in substitution of the targets genes in the background of the high aggressive strain UY031.

The mutant construction strategy it outlined in Figure 3-6. The firs step involved the amplification of a 1300-bp fragment located upstream the gen RSIPO_04290 and a 1450-bp fragment downstream the gene RSIPO_04295 from strain UY031. Both PCR products were A-T cloned into pGEM-T vector to create pGRU and pGRD and verified by sequence analysis of inserts, revealing 100% identity to the corresponding regions in strain IPO1609. In a second step, the downstream fragment was excised from pGRU and sub-cloned into pGRU next to the upstream fragment, thus creating pGRUD. Then, the Ω-spectinomycin resistance cassette was introduced into pGRUD, at a unique BamHI site located between upstream and downstream fragments, generating pGRUΩD. Restriction analysis with different enzyme combinations produced digestion products of the expected sizes, thereby confirming the accuracy of this construction (Figure 3-7). Finally, pGRUΩD was introduced into the genome of wild type *R. solanacearum* strain UY031 by natural transformation, and 17 spectinomycin resistant clones were obtained after 4 days of incubation. All clones showed typical wild-type colony morphology in TZC medium (irregular shaped, fluidal with a pink centre) and were identified as *R. solanacearum* by amplification with universal primers 759-760 (Opina et al., 1997). Deletion of the target region was verified by PCR amplification for each deleted gene using primers described in Table 3-3 and genomic DNA as template (Figure 3-7B). One resistant mutant strain, named UY031M2 was selected and used for further phenotypic evaluation.

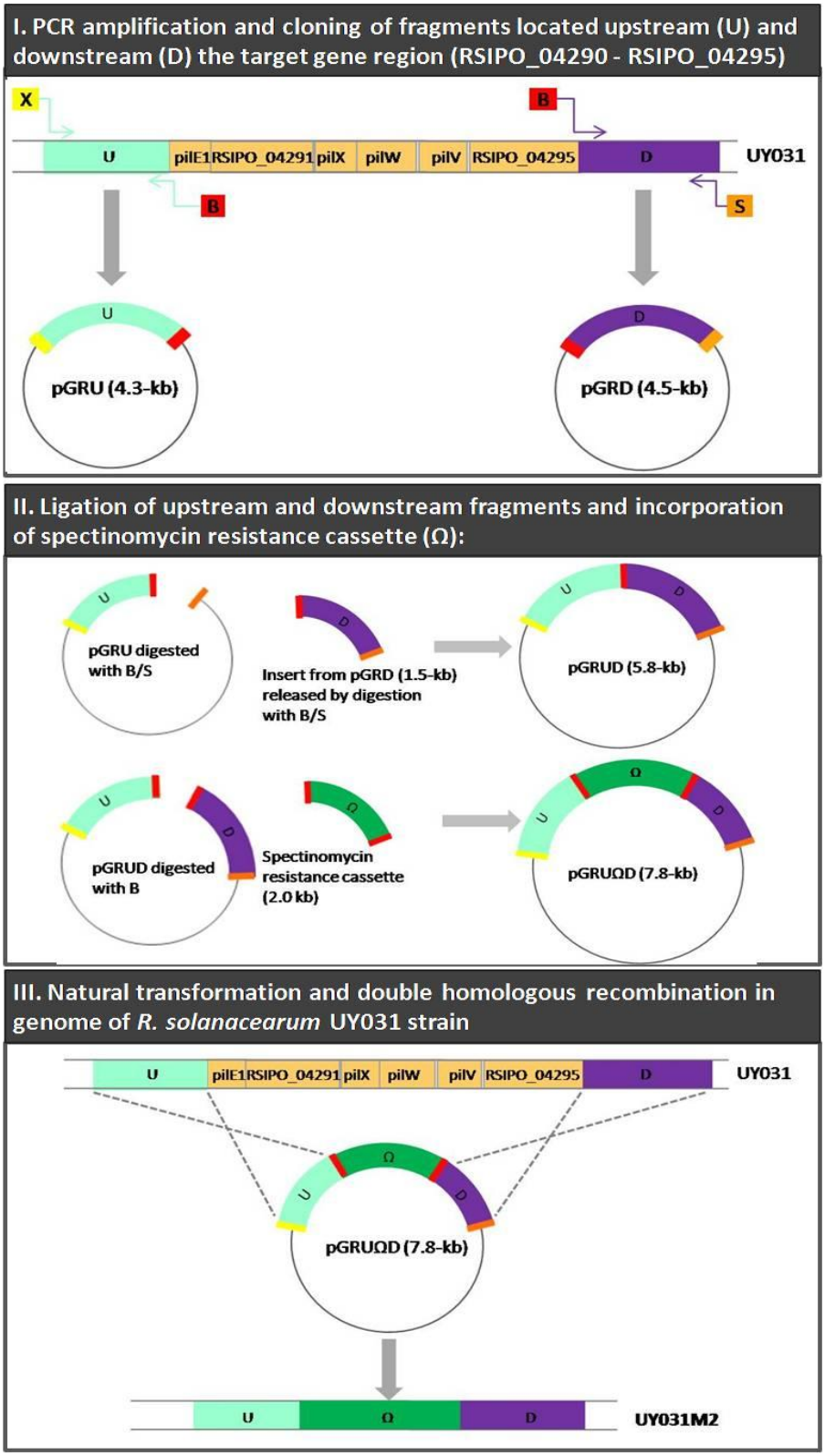


Figure 3-6. Mutant construction strategy used for deletion of gene cluster RSIPO_04290-RSIPO_04295 in *Ralstonia solanacearum* strain UY031. Panel I: specific restriction endonuclease recognition sites were incorporated in forward and reverse PCR-primers used for the amplification of fragments located upstream (U) and downstream (D) the target gene region in strain UY031. The following abbreviations were used. X (yellow): *Xba*I, B (red): *BamH*I, S (orange): *Sac*I. The resulting fragments

were cloned into pGEM-T easy, creating pGRU and pGRD. Panel II: ligation of upstream and downstream fragments followed by the incorporation of the Ω-spectinomycin resistance cassette to create pGRUΩD. Panel III: pGRUΩD was introduced into wild-type *R. solanacearum* strain UY031 by natural transformation. Incorporation of the Ω cassette in the genomic DNA was accomplished through double homologous recombination of regions of homology up- and downstream of the target genes.

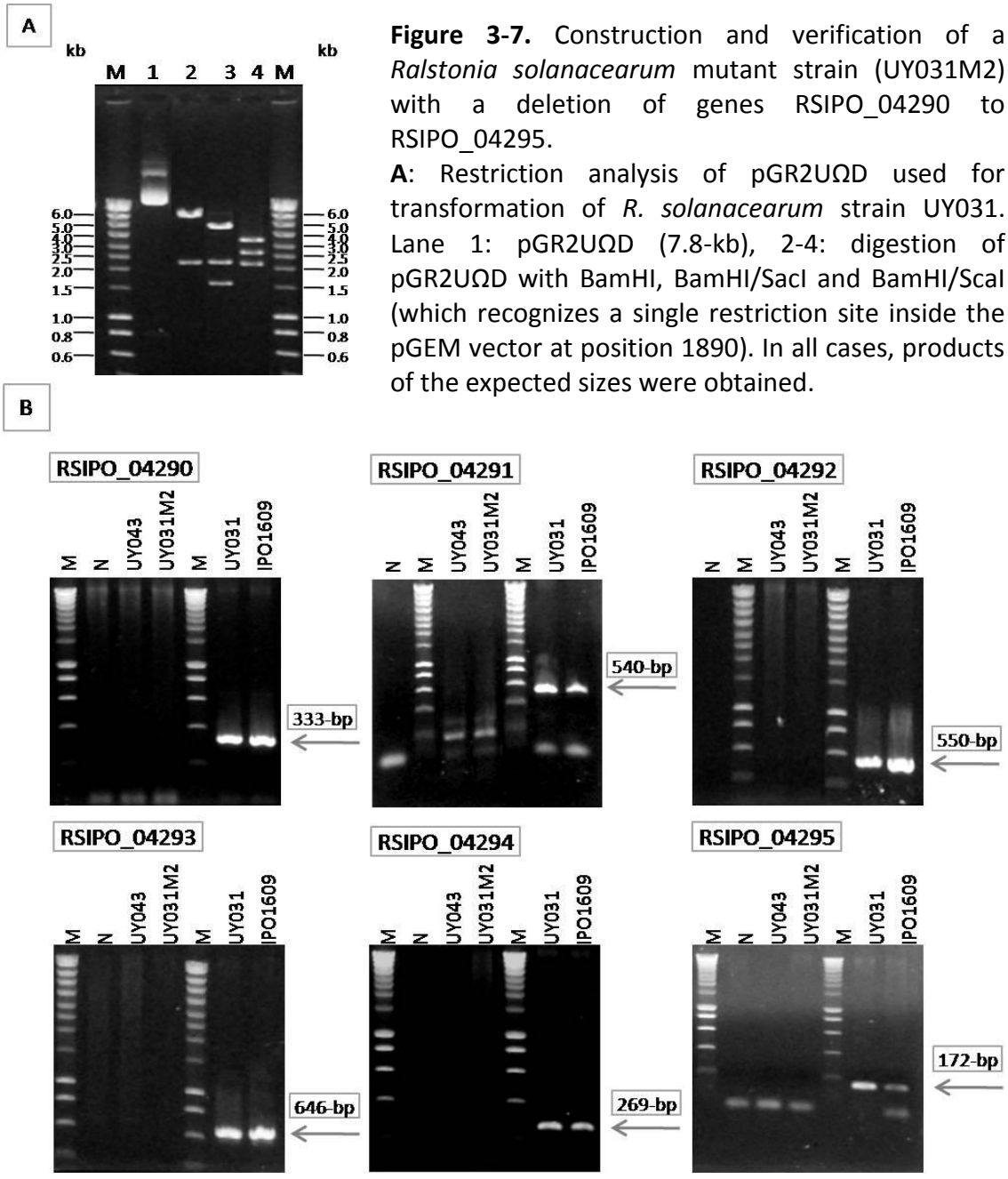


Figure 3-7. Construction and verification of a *Ralstonia solanacearum* mutant strain (UY031M2) with a deletion of genes RSIPO_04290 to RSIPO_04295.

A: Restriction analysis of pGR2UΩD used for transformation of *R. solanacearum* strain UY031. Lane 1: pGR2UΩD (7.8-kb), 2-4: digestion of pGR2UΩD with BamHI, BamHI/SacI and BamHI/ScaI (which recognizes a single restriction site inside the pGEM vector at position 1890). In all cases, products of the expected sizes were obtained.

Figure 3-7B: Verification of mutant strain UY031M2 by PCR-amplification of deleted genes RSIPO _04290 to RSIPO_04295. Reactions were performed using primers described in Table 3-3B and genomic DNA from *R. solanacearum* mutant strain UY031M2 and wild-type strains UY043, UY031, and IPO1609. M= Molecular weight marker (Smart Ladder, Eurogentec). N= Negative control.

Growth and twitching motility phenotype in culture

Mutant strain UY031M2 showed a normal mucoid colony morphology on solid media and grew as well as wild type strains UY031, UY043 and UW551 in liquid rich (CPG) and minimal (MM) medium (data not shown).

To determine if the deletion of Tfp related genes is associated with the ability to exhibit twitching motility, young colonies of wild-type and mutant strains growing on CPG plates were examined by light microscopy. Strain GMI1750 was also included for visual comparison, since it corresponds to a mutant strain with inactivation of *pilA*, which encode for the major pilin subunit in *R. solanacearum* strain GMI1000 (Kang et al., 2002). As expected, GMI1750 did not exhibit twitching motility, producing colonies with completely entire margins. In contrast, mutant strain UY031M2 showed a phenotype comparable to that of wild-type strains, producing typical micromorphologies associated with twitching motility, with layered edges and multiple irregular projections (Figure 3-8). These observations indicated that contrarily to *pilA,* the Tfp-related genes deleted in strains UY031M2 and UY043 are not essential for twitching motility in culture.

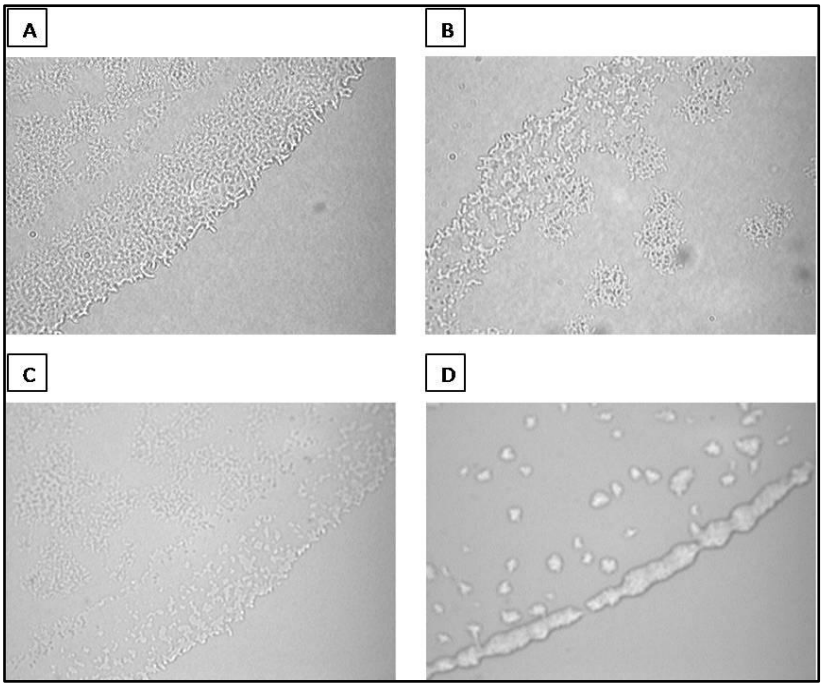


Figure 3-8. Images showing the occurrence or absence of twitching motility of *Ralstonia solanacearum* strains as seen through light microscopy after 30 h of growth on CPG plates. Typical colonies with multiple irregular projections are formed by bacteria migrating via twitching. A: strain UY031 B: strain UY043 C: strain UY031M2 (RSIPO_04390 – RSIPO_04295 mutant). D. GMI1750 (*pil*A mutant of strain GMI1000)

Aggressiveness evaluation

To determine if the cluster of Tfp-related genes, found to be deleted in strain UY043 could explain the low aggressiveness of this strain, we evaluate the aggressiveness of strain UY031M2 by inoculation of potato plants using a soil inoculation and a direct petiole inoculation assay (Table 3-5, Figure 3-9). When bacteria were directly introduced into host vascular tissue, no significant differences were observed among disease progress curves of wild-type strains UY043, UY031 and mutant strain UY031M2 (p=0,8318). Potato plants inoculated with all 3 strains began to develop wilt symptoms 4 days after inoculation, and almost all were completely dead after 12 days. In contrast, using a soil inoculation assay, which requires bacteria to locate and invade host roots from soil, significant differences were observed among the disease progress curves of the 3 strains (p<0,0001). Based on AUDPC data, mutant strain UY031M2 exhibited a reduced aggressiveness level in comparison with wild-type strain UY031, indicating that deleted genes related to Tfp are required for full pathogenicity on potato. Nevertheless, strain UY043 displayed even lower aggressiveness than mutant strain UY031M2, suggesting that additional pathogenicity determinants may explain the differences in aggressiveness between these strains. Interestingly, for both UY031M1 and UY043 strains, appearance of symptoms was delayed and leaf wilting was observed just after 13 days, while for strain UY031 first symptoms appeared 5 days after inoculation.

Table 3-5. Aggressiveness evaluation of wild-type and mutant *Ralstonia solanacearum* strains on potato (cv. Chieftain).

Strain	**AUDPC**[a]	
	Direct petiole inoculation	**Soil inoculation**
UY031	48.8 A	37.0 A
UY043	46.4 A	12.0 B
UY031M2	46.8 A	21.5 C

[a] AUDPC: area under the disease progress curve based on average wilting scoring. Values followed by the same letter in the same column are not significantly different (Tukey's multiple comparison test, p = 0.05)

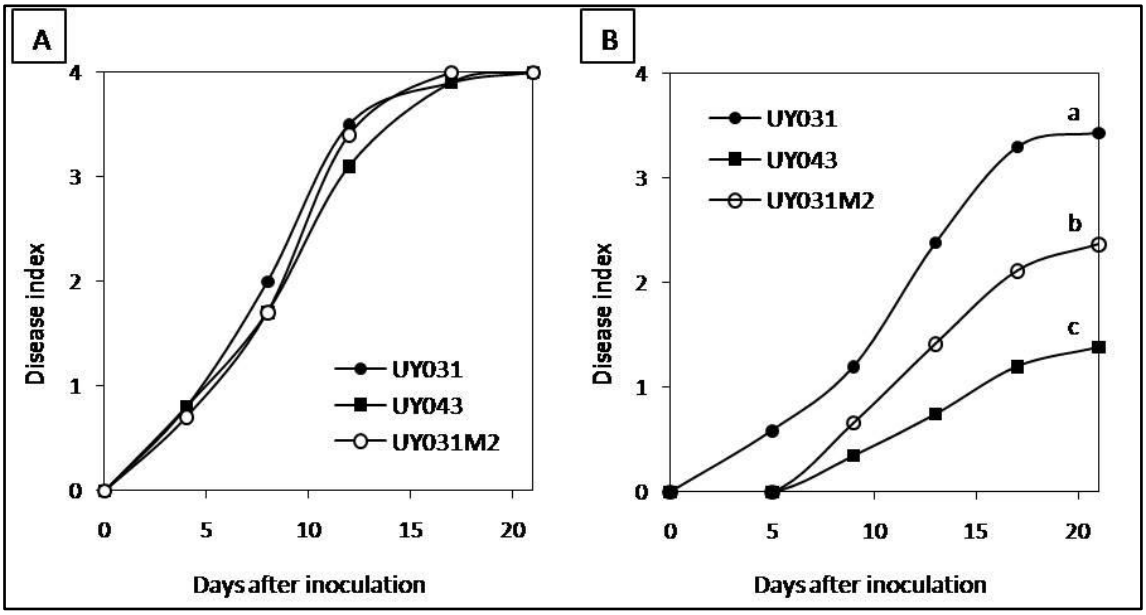


Figure 3-9. Disease progress curves of *Ralstonia solanacearum* strains on potato (cv. Chieftain). Wild-type strains UY031 (black circles), UY043 (black squares), and mutant strain UY031M2 (white circles) were tested by two different inoculation methods. A. Direct petiole inoculation assay: plants were inoculated by introducing about 1000 cells into the cut petiole of the third true leaf from the top of the plant. B. Soil inoculation assay: plants were inoculated by pouring bacterial suspensions into the soil to a final concentration of 5 x 10^6 cfu/g soil. Diseased development was recorded for 21 days at regular time intervals on a disease index scale ranging from 0 to 4. Each point represents the mean disease index for two independent experiments with 5 and 3 replicates for petiole and soil inoculation methods respectively. Letters indicate different levels of strain aggressiveness according to the area under disease progress curve based on average disease index (AUDPC) (Tukey's test, p = 0.05)

Colonization assays

An in vitro root inoculation assay was carried out to evaluate the ability of *R. solanacearum* strains to invade the roots and colonize potato plants. This assay allowed standardized and repeatable conditions to facilitate the comparison of bacterial densities, and was applied to study colonization fitness of mutant strain UY031M2 and for comparison with wild-type *R. solanacearum* strains UY031 and UY043. In vitro plantlets growing in vertically oriented squared petri dishes were inoculated by applying the bacterial suspension along the entire length of the roots. Population dynamics of each strain was evaluated by determining the internal bacterial densities in roots and stems sections at different sampling times by dilution plating in the appropriate medium (Table 3-6, Figure 3-10).

At one day after inoculation (DAI), there was no significant difference between roots (p=0.8692) and stems populations (p=0.8493) of wild-type and mutant strains. Plants inoculated with strain UY031, showed a rapid increase in bacterial densities, in agreement with the high aggressiveness level observed previously for this strain. Bacterial measurements 4 DAI revealed a significant reduction in the colonization ability of strain UY043 and UY031M2, reaching a population approximately 30-fold lower than strain UY031, for both root (p=0.0003) and stem (p=0.003) tissues. The internal bacterial densities at 7 DAI showed a correlation with aggressiveness levels of strains measured by wilting scoring using soil inoculation procedure, where mutant strain UY031M2 exhibited an intermediate response between strain UY031 and UY043. In stem tissue at 7 DPI, differences in bacterial populations were more pronounced and significant reductions of 200-fold for strain UY031M2 and 2000-fold for UY043 were detected in comparison with strain UY031 (p=0.0001). In root tissue, strain UY043 reached a population about 60-fold lower than strain UY031 (p=0.0104) and mutant strain UY031M2 showed an intermediate response, but the differences with both wild-type strains were not statistically significant.

Table 3-6. Internal bacterial densities of *Ralstonia solanacearum* strains UY031, UY043, and UY031M2 in potato roots (R) and stems (S) sections measured 1, 4 and 7 days after root inoculation (DAI) using an in vitro assay.

Strain	**Bacterial densities (cfu/g plant tissue)[a]**					
	1 DAI		**4 DAI**		**7DAI**	
	R	**S**	**R**	**S**	**R**	**S**
UY031	$4.2x10^3$ A	$1.5x10^3$ A	$4.1x10^5$ A	$2.9x10^6$ A	$1.3x10^7$ A	$2.5x10^9$ A
UY043	$3.6x10^3$ A	$1.3x10^3$ A	$1.7x10^4$ B	$7.8x10^4$ B	$2.2x10^5$ AB	$1.3x10^6$ B
UY031M2	$3.9x10^3$A	$1.1x10^3$ A	$1.1x10^4$ B	$1.4x10^5$ B	$1.3x10^6$ B	$1.3x10^7$ C

[a] Values followed by the same letter in the same column are not significantly different (Tukey's multiple comparison test, p=0.05)

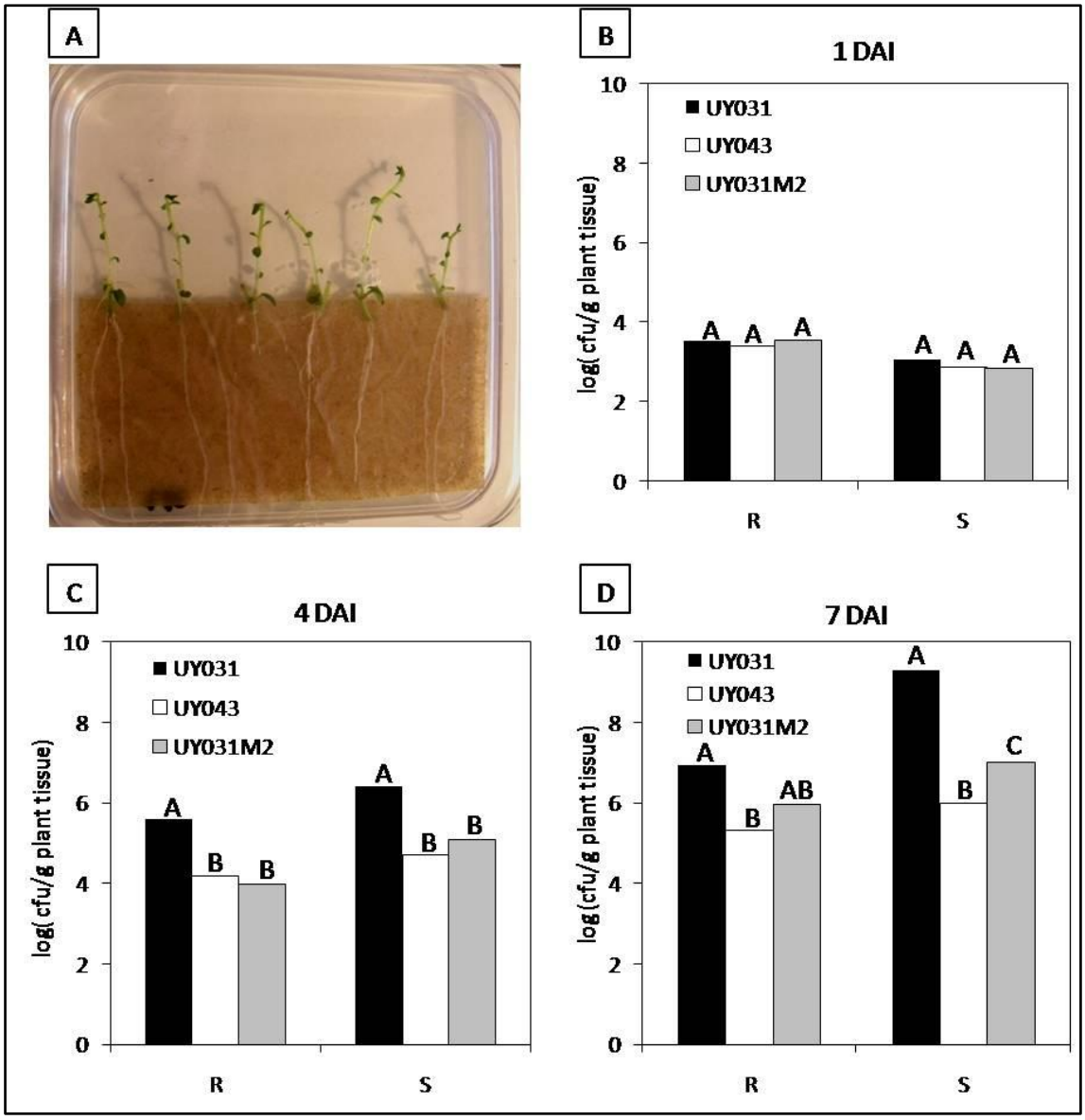


Figure 3-10. Population dynamics of *Ralstonia solanacearum* strains UY031, UY043, and UY031M2 on potato (cv. Chieftain). A: Presentation of the system used for the *in vitro* root inoculation assay. Potato plantlets growing in squared Petri dishes were inoculated by applying bacterial suspensions of 10^7cfu/ml along the roots. For each strain, 3 replicate Petri dishes were sampled 1 (B), 4 (C) and 7 (D) days after inoculation (DAI), and the plantlets were pooled and processed for internal bacterial density counts in root (R) and stem (S) sections. Different letters on top of columns within each evaluation time and plant section indicate significant differences between strains (Tukey's multiple comparison test, p=0.05)

DISCUSSION

In this study, we used CGH on a spotted microarray reflecting the pan-genome of *R. solanacearum* to compare the gene content of a set of Uruguayan strains exhibiting different aggressiveness levels in order to identify candidate pathogenicity genes. Several previous studies revealed low genetic variation among IIB-1 *R. solanacearum* strains using fingerprinting genetic markers and sequence analysis of specific genes (Castillo and Greenberg, 2007; Poussier et al., 2000; Timms-Wilson et al., 2001; Van der Wolf et al., 1998). In support, the draft genomes of two 2 strains from this group (UW551 and IPO1609) are now available revealing almost an identical sequence and genome structure (Gabriel et al., 2004; Guidot et al., 2009b). Interestingly, in this study we were able to detect variation among IIB-1 *R. solanacearum* strains, highlighting the CGH-microarray technology as a powerful way to perform whole genomic comparisons, even among closely related strains. Furthermore, in this approach the low genetic variation among this group could be considered advantageous, since this allowed us to identify a limited number of differential genes, therefore enhancing the probability of finding a correlation between genome composition and a particular phenotype.

In a first attempt, a comparison was made between the genetic content of groups of strains with differences in aggressiveness on several hosts including tomato, potato and the wild potato relative *S. commersonii*. This analysis led us to identify a list of 9 candidate genes found to be present in 6 high aggressive strains and absent from 3 low aggressive strains. However, none of these genes was validated since discrepancies were found between microarray assignments as present or absent based on a cut-off ratio, compared to the PCR method. Different reasons could explain these results. Microarrays detect homology over the whole length of the probe, which is about 70-bp long, whereas PCR detects homology to 2 short sequences that are spaced at a conserved distance from each other. For candidate genes detected in this study, the microarray probes were designed based on the sequence of strain GMI1000, while most PCR primers were designed based on the sequence of the corresponding homologous gene in strain IPO1609, which is more related to Uruguayan strains. Thus,

false negative results (genes considered as absent by CGH but with positive amplification by PCR) could be attributed to the existence of sufficiently divergence between the sequence of the gene in the tested strain and the sequence of the reference strain spotted on the microarray. On the other hand, for some genes no amplification product was obtained, suggesting the occurrence of false positives probably explained by a cross-hybridization between genes with high similarity. In addition, all tested strains were amplified using universal primers for *R. solanacearum* as a positive control of amplification, thereby confirming the identity of the strains and the reliability of our PCR results. Based on these results, we failed to identify candidate genes associated with differences in aggressiveness among Uruguayan *R. solanacearum* strains using a CGH-microarray approach. This may indicate that allelic variations, rather than genomic content per se, contribute to the divergence in disease-causing ability between these strains, since such variations would not be detected using CGH. However this approach was selected since it was previously shown a high variation in gene distribution in *R. solanacearum*, including among known pathogenicity determinant such as type III effectors (Guidot et al., 2007; 2009a).

A second genomic comparison was performed in order to identify specific differences in gene content of strain UY043. This strain was selected because it was isolated from soil, unlike the other strains that were isolated from potato tubers with typical bacterial wilt symptoms. In addition, this strain consistently displayed very low aggressiveness on different hosts, as previously described in Chapter 2. Based on the particular traits of strain UY043, a comparative analysis was undertaken in order to allow insight in the putative genomic changes incurred in a strain from the open environment, which may explain their low level of aggressiveness. A major finding resulted from this analysis was the occurrence of a deletion in strain UY043 covering a 33.7-kb region of the megaplasmid designated as DR043. Validation by PCR amplification confirmed that all the 25 genes contained in this region were absent in strain UY043 and present in 12 *R. solanacearum* strains from Uruguay, suggesting that this deletion event occurred exclusively in the environmental strain UY043.

Genomic rearrangements such as deletions, inversions, duplications and transpositions have already been reported to occur naturally in *R. solanacearum* as well as in many other bacteria (Stevens and van Elsas, 2010). These events are usually induced by the presence of insertion sequences, and recombination hot spot elements which are known to be scattered throughout the *R. solanacearum* genome (Salanoubat et al., 2002). Together with the ability to acquire new genetic information from other strains or species by horizontal gene transfer, these genomic rearrangements are responsible of the genome evolution and diversification of the *R. solanacearum* species complex. In order to determine a possible deletion mechanism in strain UY043, the region DR043 was analyzed based on sequence information from strain IPO1609 draft genome. Interestingly, high homology was found between the sequences covering both borders of the region, which included two partial transposase sequences of external origin. It seems likely, that this feature promotes the occurrence of homologous recombination between both sequences, leading to deletion of a DNA loop of 33.7-kb in strain UY043. This hypothesis could be explored by analyzing the sequence generated at the junction site in strain UY043.

Recently, similarly spontaneous deletion was reported by Guidot et al. (2009a), who applied the same CGH approach used in this study to investigate the extent of horizontal gene transfers and genomic rearrangements occurred after natural transformation between different *R. solanacearum* strains. One of the genomic changes detected in this study, was the loss of a cluster of 29.3-kb harboring 27 genes located within the megaplasmid. Interestingly, this DNA block is partially overlapping with the deleted DR043 region from strain UY043, suggesting that this region might be subjected to a high frequency of genomic rearrangements.

Comparison of DR043 region from strain IPO1609 with the corresponding regions in *R. solanacearum* strains belonging to different phylotypes, also revealed the occurrence of genomic rearrangement within this region during evolution. On the other hand, high conservation regarding gene content and sequence homology was found between phylotype II strains.

Another deletion event in *R. solanacearum* has been recently reported by Stevens and van Elsas (2010), who used suppressive subtractive hybridization to compare a potato

R. solanacearum IIB-1 strain with a related strain isolated from a water course. Following this approach, they identified a deletion of a putative genomic island of 17.6-kb in the environmental strain. This region, denoted PGI-1, is flanked by a composite of 2 insertion sequences elements, shows a low average G+C content, and has several features that support the hypothesis that it constitutes a mobile genomic island. PGI-1 harbors two genes of potential ecological relevance, one encoding a hypothetical protein with a RelA/SpoT domain and the other, a putative cellobiohydrolase. Phenotypic characterization indicates that this environmental strain is affected in growth rate, response to cold stress and invasion of tomato, suggesting the role of this genomic island in the adaptation of the strain to local conditions (Stevens et al., 2010).

Analysis of annotation data for genes located within the region DR043 strongly suggested that the loss of this region is associated with the low aggressiveness found in strain UY043. In this study, we focused the analysis on a cluster of 6 genes involved in Tfp biogenesis. Tfp are widely distributed surface structures that mediate an extraordinary array of functions, including adhesion, biofilm formation, DNA uptake and twitching motility (Pelicic, 2008). It has been shown that these appendages also promote the attachment to solid surfaces, including host tissues, and are considered as important virulence factors in many pathogenic bacteria (Pizzarro-Cerdá and Cossart, 2006). In contrast to the well-established role of Tfp in the pathogenicity of animal-pathogenic bacteria, their role in plant-pathogenic bacteria is poorly understood. Analysis of strain IPO1609 genome identified about 40 genes that encode putative proteins involved in the biogenesis and function of Tfp, but to date little information exists regarding the function for most of these genes. Previous studies reported that Tfp in *R. solanacearum* are involved in twitching motility, biofilm formation, and virulence (Kang et al. 2002; Liu et al. 2001). However, the role of Tfp in colonization of host plants and pathogenesis has been studied only on tomato plants and using a limited number of strains belonging to phylotype I. The major pilin in strain GMI1000 was identified as a 17 kDa protein designated PilA, based on the nomenclature adopted for *Pseudomonas aeruginosa* (Kang et al., 2002). Two additional proteins, PilQ and PilT, were studied in *R. solanacearum* based on its conserved sequences and function in other species. These proteins were proved to be involved in biogenesis of

functional Tfp, since *pilA, pilQ*, and *pilT* mutants did not exhibited twitching motility and were reduced in autoaggregation, biofilim formation and caused slower disease development, and less severe wilting symptoms in tomato plants (Kang et al. 2002; Liu et al. 2001). The functions of Tfp and their role in plant – pathogen interactions were also studied in other plant pathogens including *Xanthomonas oryzae, Xilella fastidiosa,* and *Acidovorax citrulli* (Bahar et al., 2009; Li et al., 2007; Wang et al., 2007).

In this study, a cluster of 6 genes involved in Tfp biogenesis were identified within the DR043 region. This gene cluster showed identical distribution and high sequence conservation in all sequenced *R. solanacearum* strains. Furthermore, almost the same organization is found in other bacteria species, suggesting that the molecular mechanisms underlying biogenesis and functions of Tfp are conserved (Mattick, 2002; Pelicic, 2008). Based on the nomenclature adopted for *P. aeruginosa*, 4 of these genes were annotated in *R. solanacearum* as *pilE1*, *pilX*, *pilW* and *pilV*. In addition, 2 Tfp related genes are included in the cluster: RSIPO_04291 which encodes for a protein related to PilY1 and RSIPO_04296, encoding for a putative Tfp-related pilin. The corresponding cluster in *P. aeruginosa* contained 8 genes (*pilE*, *pilY2*, *pilY1*, *pilV*, *pilW*, *pilX*, *fimT* and *fimU*). These proteins were designated as minor pilin-like proteins due to their limited abundance relative to the major pilin PilA (Koomey, 1995). Many studies addressed the importance of minor pilins for biogenesis of functional pili, however the role of these proteins may be variable among different model bacteria. In pathogenic *Neisseria* species these proteins modulate various Tfp-linked functions, like DNA uptake, bacterial aggregation and adhesion, but were found to be dispensable for Tfp biogenesis (Helaine et al., 2007). However, in *P. aeruginosa* it was proposed that minor pilins are involved in the initiation of pilus assembly and are supposed to be incorporated into the growing fiber, playing an essential role in biogenesis of functional Tfp (Giltner et al., 2010).

In this study we were interested to know whether the deletion of this cluster of Tfp related genes could explain the low aggressiveness of *R. solanacearum* strain UY043, in order to investigate the role of minor pilins in *R. solanacearum.* For this purpose, a mutant strain was constructed by deleting the corresponding gene cluster in the background of the high aggressive *R. solanacearum* strain UY031. The resulting mutant

strain (UY031M2) was evaluated based on their phenotype and twitching motility in culture and their aggressiveness and colonization ability on potato plants.

Twitching motility involves the extension and retraction of the pilus through assembly and disassembly of pilin subunits at the inner membrane, and consequent movement of the bacterium along a surface (Mattick, 2002). In this study we evaluate this ability in culture by the observation of the typical twitching phenotype of young colonies exhibiting layered edges and multiple irregular projections. As previously reported, twitching motility was not detected in a mutant strain that do not produce the major pilin (*pilA*$^{-}$), since this protein is absolutely required for piliation in *R. solanacearum* (Kang et al., 2002). In contrast, our observations suggest that both wild-type strain UY043 and mutant strain UY031M2 retain the ability to produce twitching motility in culture, indicating that the deleted genes in these strains are not essential for pili assembly and retraction. However, this conclusion is based on preliminary assays and the occurrence of subtle effects on twitching motility might have not been detected. Therefore, it would be interesting to conduct a more exhaustive evaluation, by comparing the zone of twitching of wild-type and mutant strains, to assess whether the deletion of these genes has a quantitative effect on this activity. In addition, the examination by electron microscopy will contribute to determine the amount and distribution of Tfp in wild-type and mutant strains.

The ability to invade roots, establish an infection, colonize the plant, and produce virulence factors is necessary for *R. solanacearum* to cause disease in susceptible hosts. Multiple pathogenicity traits with a myriad of functional roles are involved in this complex, dynamic and changeable phenomenon of interaction with the plant host. We previously found that strain UY043 displayed a reduced aggressiveness assessed by soil inoculation on several hosts, including tomato, potato and the wild potato relative *S. commersonii*. However, when plant inoculation was performed by placing the bacteria inside the stem, wilt symptoms were observed in a few days and no differences were detected from other *R. solanacearum* high aggressive strains (see Chapter 2). To investigate if the cluster of Tfp related genes found to be deleted in strain UY043, could explain this particular behavior, we used both inoculation methods to evaluate the aggressiveness of mutant strain UY031M2 on potato plants.

Using a biologically representative soil inoculation assay that required bacteria to actively find and invade host plant roots from the soil, strain UY031M2 caused significantly less disease than its wild-type parent indicating that deleted genes related to Tfp are required for full pathogenicity on potato. Nevertheless, strain UY043 displayed even lower aggressiveness than mutant strain UY031M2, suggesting that additional pathogenicity determinants may explain the differences in aggressiveness between these strains. In addition, we concluded that the low aggressiveness of both UY031M2 and UY043 strains is not due to reduced growth rates, since both strains grew as well as the high aggressive strain UY031 either in rich or minimal medium.

It has been shown previously that elimination of Tfp pilin synthesis by inactivating *pilA* produced reduced virulence when both unwounded roots and wounded petioles of tomato plants were inoculated. Therefore it was proposed that piliation could contribute to pathogenesis during both the invasion of roots and when the bacterium is inside plants (Kang et al., 2002). However, in our study, when strains were inoculated directly into the vascular system through a cut leaf petiole, the mutant and wild-type strains caused the same level of disease on potato plants. This finding suggests that once bacteria achieves the vascular system the Tfp-related genes that are deleted in mutant strain UY031M2 are no longer needed for disease development on potato. Interestingly, when soil inoculation was performed, a delayed appearance of symptoms was observed in plants inoculated with both UY031M2 and UY043 strains in comparison to the high aggressive strain UY031. Together these results suggest that minor pilins encoded by these particular Tfp-related genes mainly contribute to the early bacterial wilt pathogenesis, probably during the attachment to and/or colonization of potato roots.

To test this second hypothesis, we applied an *in vitro* root inoculation assay to study colonization fitness of mutant strain UY031M2 on potato plants. Interestingly, internal bacterial measurements in roots and stem tissues at 4 DAI revealed a similar reduction in the colonization ability for strains UY043 and UY031M2 in comparison with strain UY031. This observation indicates that the reduced colonization fitness of strain UY043 could be attributed to the deletion of the cluster of Tfp-related genes located in the DR043 region. It seems likely that these genes contribute to Tfp biogenesis and/or

function, thus reducing pathogenesis during invasion of roots by affecting adherence to roots surfaces and migration to wound sites for colonization. However, further studies are necessary to explore the connection between these deleted genes and other Tfp associated activities like biofilm formation, surface adherence and cell aggregation, in order to clarify its contribution to colonization and aggressiveness on potato. On the other hand, at 7 DAI mutant strain UY031M2 exhibited an intermediate response between strain UY031 and UY043, in agreement with previous observations based on wilt symptoms development after soil inoculation. This finding suggests that additional pathogenicity determinants are involved in the low aggressiveness observed for strain UY043. Nevertheless, the functional analysis conducted in this study is preliminary and should be completed with a complementation analysis of strain UY031M2. This analysis will allow us to verify whether the observed effect on the aggressiveness of *R. solanacearum* can be attributed to the Tfp-related genes.

Interestingly, 2 genes located within the region DR043 encoded for candidate effectors proteins secreted through the type three secretion system (T3SS). This secretion system plays a central role in the pathogenesis of *R. solanacearum* and many other plant and animal bacterial pathogens (Cornelis and Van Gijsegem, 2000; Galán and Collmer, 1999; He et al., 2004). Extensive studies were conducted to identify the repertoire of effector proteins (T3E) translocated into plant cells through the T3SS and today a refined list of 94 T3E is recognized (reviewed in Poueymiro and Genin, 2009). Both candidate genes identified in this study (RSIPO_04287 and RSIPO_04310) are broadly conserved within the different strains of the species and have been demonstrated to be secreted in a T3SS-dependent manner in *R. solanacearum* (Mukaihara and Tamura, 2009; Tamura et al., 2005). The gene RSIPO_04287, share homology with the known T3E HopAA1-1 of *P.syringae* pv. tomato, and was found to be directly regulated by HrpB, an AraC-type transcriptional activator responsible for the expression of T3SS and T3E genes in *R. solanacearum* (Mukaihara et al., 2004; Occhialini et al., 2005). The gene RSIPO_04310 belongs to the SKWP effector family, which contain a novel amino acid repeat domain related to the heat/armadillo repeats from eukaryotes known to mediate protein–protein interactions. It was proposed that these proteins might interact with plant factors via the SKWP repeat domain to exert

virulence functions (Mukaihara and Tamura, 2009). Based on this information it seems likely that both candidate genes could contribute to bacterial wilt development and their role in the pathogenesis of *R. solanacearum* should be further studied. To address this issue, a preliminary approach should be to perform a complementation analysis of strain UY043 with plasmids harboring the effectors genes identified in DR043.

In summary, this study provides insights into the pathogenesis of *R. solanacearum*, in particular with regards to IIB-1 strains and the interaction with potato as its natural host. The application of CGH using a microarray chip reflecting the pan-genome of *R. solanacearum* proved to be a powerful tool to perform whole genomic comparisons between strains with differences in aggressiveness and source of isolation. This approach allowed us to identify the occurrence of a deletion of a 33.7-kb region in an environmental strain with strongly reduced aggressiveness. Analysis of this region denoted as DR043 strongly suggested that this genomic rearrangement is associated with the low aggressiveness found in strain UY043 and offers the potential to identify candidate pathogenicity genes. In this study, we focused the analysis on a cluster of 6 genes involved in Tfp biogenesis. The functional analysis performed suggests that minor pilins encoded by this genetic cluster mainly contribute to the early bacterial wilt pathogenesis and the colonization fitness of potato roots, although they appeared to be not essential for twitching motility in culture. Future studies are necessary to investigate the involvement of these proteins in other Tfp-associated activities like biofilm formation, surface adherence and cell aggregation. The individual analysis of each Tfp-related protein would contribute to elucidate the role of minor pilins in *R. solanacearum* and to fully understand its contribution to host attachment, colonization and disease development. The results generated in this study also suggest that additional genes located within the region DR043 are involved in the low aggressiveness observed for strain UY043. Further work focused on the identified candidate genes will attempt to more precisely establish this hypothesis. Finally, this study highlights the occurrence of genomic rearrangements in *R. solanacearum*, which are known to play an important role in the evolution and diversification of the species. Further examination of the genetic make-up of a larger collection of strains from different sources of isolation could contribute to understand the importance of these

phenomena in the potential adaptation of *R. solanacearum* strains to selective pressures from the environment.

REFERENCES

Arlat M.C., Gough L., Zischek C., Barberis P.A., Trigalet A. and Boucher C.A. 1992. Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. Mol. Microbiol. 6:3065–3076

Ausubel F.H., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., and Struth K. 1994. Current Protocols in Molecular Biology. John Wiley and Sons, Canada.

Bahar O., Goffer T., and Burdman S. 2009. Type IV pili are required for virulence, twitching motility, and biofilm formation of *Acidovorax avenae* subsp. *citrulli.* Mol. Plant-Microbe Interact. 22(8):909-920.

Bertolla F., Van Gijsegem F., Nesme X., Simonet P. 1997. Conditions for natural transformation of *Ralstonia solanacearum*. Appl. Environ. Microbiol. 63:4965–4968.

Boucher C., Martinel A., Barberis P., Alloing G., Zischek C. 1985. Virulence genes are carried by a megaplasmid of the plant pathogen *Pseudomonas solanacearum*. Mol. Gen. Genet. 205:270–275.

Buddenhagen I. and Kelman A.. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 2:203–230.

Brown D.G. and Allen C. 2004. *Ralstonia solanacearum* genes induced during growth in tomato: an inside view of bacterial wilt. Mol. Microbiol. 53:1641–1660.

Castillo J.A. and Greenberg J.T. 2007. Evolutionary dynamics of *Ralstonia solanacearum*. Appl. Environ. Microbiol. 73: 1225–1238.

Cornelis G.R. and Van Gijsegem F. 2000. Assembly and function of type III secretory systems. Annu. Rev. Microbiol. 54: 735–774.

Coutinho T.A. 2005. Introduction and prospectus of the survival of *R. solanacearum*. Pages 29-38 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A. C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Di Rienzo J.A., Casanoves F., Balzarini M.G., Gonzalez L., Tablada M., and Robledo C.W. InfoStat versión 2009. Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Argentina.

Elphinstone J.G. 2005. The current bacterial wilt situation: a global overview. Pages 9-28 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A. C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Fegan M. and Prior P. 2005. How complex is the '*Ralstonia solanacearum*' species complex? Pages 449- 461 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A. C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Gabriel D.W., Allen C., Schell M., Denny T.P., Greenberg J.T., Duan Y.P., Flores-Cruz Z., Huang Q., Clifford J.M., Presting G., González E.T., Reddy J., Elphinstone J., Swanson J., Yao J., Mulholland V., Liu L., Farmerie W., Patnaikuni M., Balogh B., Norman D., Alvarez A., Castillo J.A., Jones J., Saddler G., Walunas T., Zhukov A., and Mikhailova, N. 2006. Identification of open reading frames unique to a select agent: *Ralstonia solanacearum* race 3 biovar 2. Mol. Plant-Microbe Interact. 19:69-79.

Galán J.E. and Collmer A. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284: 1322–1328.

Genin S. and Boucher C. 2004. Lessons learned from the genome analysis of *Ralstonia solanacearum*. Annual Review of Phytopathology. 42:107–134.

Giltner C.L., Habash M., and Burrows L.L. 2010. *Pseudomonas aeruginosa* minor pilins are incorporated into type IV pili. J. Mol. Biol. 398: 444–461.

González E.T. and Allen C. 2003. Characterization of a *Ralstonia solanacearum* operon required for polygalacturonate degradation and uptake of galacturonic acid. Mol. Plant-Microbe. Interact. 16: 536–544.

Guidot A., Prior P., Schoenfeld J., Carrere S., Genin S., Boucher C.A. 2007. Genomic structure and phylogeny of the plant pathogen *Ralstonia solanacearum* inferred from gene distribution analysis. J. Bacteriol. 189:377–387.

Guidot A., Coupat B., Fall S., Prior P., and Bertolla F. 2009a. Horizontal gene transfer between *Ralstonia solanacearum* strains detected by comparative genomic hybridization on microarrays. ISME J. 3:549–562.

Guidot A., Elbaz M., Carrere S., Siri M.I., Pianzzola M.J., Prior P. and Boucher C. 2009b. Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathology 99:1105–1112.

Hayward A.C. 1964. Characteristcs of *Pseudomonas solanacearum*. Journal of Appl. Bacteriol. 27:265–77.

Hayward A.C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29:65–87.

Hayward A.C. 1994a. Systematics and phylogeny of *Pseudomonas solanacearum* and related bacteria. Pages 123-135 in: Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A.C. Hayward and G.L. Hartman, eds. CAB International, Wallingford, UK.

Hayward A.C. 1994b. The hosts of *Pseudomonas solanacearum*. Pages 9-24 in: Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A.C. Hayward and G.L. Hartman, eds. CAB International, Wallingford, UK.

He S.Y., Nomura K. and Whittam T.S. 2004. Type III protein secretion mechanism in mammalian and plant pathogens. Biochim. Biophys. Acta 1694, 181–206.

Helaine S., Dyer D.H., Nassif X., Pelicic V., and Forest K.T. 2007. 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. Proc. Natl. Acad. Sci. USA 104: 15888–15893.

Hendrick C. and Sequeira L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. Appl. Environ. Microbiol. 48:94–101.

Kang Y., Liu H., Genin S., Schell M.A., and Denny T.P. 2002. *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. Mol. Microbiol. 46:427-437.

Kelman A., 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693–695.

Koomey M. 1995. Prepilin-like molecules in type 4 pilus biogenesis: minor subunits, chaperones or mediators of organelle translocation? Trends Microbiol. 3(11):409-10.

Li Y., Hao G., Galvani C.D., Meng Y., De La Fuente L., Hoch H.C., and Burr T.J. 2007. Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation, and cell-cell aggregation. Microbiology 153:719-726.

Lin Y.-M., Chou I.-C., Wang J.-F., Ho F.-I., Chu Y.-J., Huang P.-C-, Lu D.-K., Shen H.-L-, Elbaz M., Huang S.-M. and Cheng C.-P. 2008. Transposon mutagenesis reveals differential pathogenesis of *Ralstonia solanacearum* on tomato and Arabidopsis. Mol. Plant-Microbe. Interact. 21(9):1261–1270.

Liu H., Kang Y., Genin S., Schell M.A., and Denny T.P. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiology 147:3215-3229.

Liu H., Zhang S., Schell M.A., Denny T.P. 2005. Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol. Plant-Microbe. Interact. 18: 1296–1305.

Mattick J.S. 2002. Type IV pili and twitching motility. Annu. Rev. Microbiol. 56:289–314.

Montanelli C., Chiari A., Chiari T., Stefanini F., and Nascari G. 1995. Evaluation of resistance to *Pseudomonas solanacearum* in potato under controlled conditions. Euphytica 81:35-43.

Mukaihara T., Tamura N., Murata Y., and Iwabuchi, M. 2004. Genetic screening of Hrp type III-related pathogenicity genes controlled by the HrpB transcriptional activator in *Ralstonia solanacearum*. Mol. Microbiol. 54:863–875.

Mukaihara T. and Tamura N. 2009. Identification of novel *Ralstonia solanacearum* type III effectors proteins through translocation analysis of hrpB-regulated gene products. Microbiology 155:2235–2244.

Occhialini A., Cunnac S., Reymond N., Genin S. and Boucher C.A. 2005. Genomewide analysis of gene expression in *Ralstonia solanacearum* reveals that the *hrpB* gene acts as a regulatory switch controlling multiple virulence pathways. Mol. Plant-Microbe. Interact. 18:938–949.

Opina N., Tavner F., Hollway G., Wang J.-F., Li T.-H., Maghirang R., Fegan M., Hayward, A.C., Krishnapillai V., Hong W.-F., Holloway B.W., and Timmis J.N. 1997.A novel method for development of species and strain specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). Asia-Pac J. Mol. Biol. 5:19-30.

Pelicic V. 2008. Type IV pili: e pluribus unum? Mol. Microbiol. 68(4):827–837.

Pizarro-Cerdá J. and Cossart P. 2006. Bacterial adhesion and entry into host cells. Cell 124:715–727.

Poueymiro M. and Genin S. 2009. Secreted proteins from *Ralstonia solanacearum*: a hundred tricks to kill a plant. Curr. Opin.Microbiol. 12:44–52

Poussier S., Trigalet-Demery D., Vandewalle P., Goffinet B., Luisetti J., and Trigalet A. 2000. Genetic diversity of *Ralstonia solanacearum* as assessed by PCR-RFLP of the hrp gene region, AFLP and 16S rRNA sequence analysis, and identification of an African subdivision. Microbiology 146:1679–1692.

Prentki P. and Kirsch H.M. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29,:303-313.

Remenant B., Coupat-Goutaland B., Guidot A., Cellier G., Wicker E., Allen C., Fegan M., Pruvost O., Elbaz M., Calteau A., Salvignol G., Mornico D., Mangenot S., Barbe V., Médigue C. and Prior P. 2010. Genomes of three tomato pathogens within the *Ralstonia*

solanacearum species complex reveal significant evolutionary divergence. BMC Genomics, 11:379

Salanoubat M., Genin S., Artiguenave F., Gouzy J., Mangenot S., Arlat M., Billault A., Brottier P., Camus J.C., Cattolico L., Chandler M., Choisne N., Claudel-Renard C., Cunnac S., Demange N., Gaspin C., Lavie M., Moisan A., Robert C., Saurin W., Schiex T., Siguier P., Thebault P., Whalen M., Wincker P., Levy M., Weissenbach J. and Boucher C.A. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497-502.

Sambrook J., Russell D.W. 2001. Molecular Cloning: A laboratory manual. 3rd ed, Cold Spring Harbor Laboratory Press, New York.

Saile E., McGarvey J.A., Schell M.A., and Denny T.P. 1997. Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. Phytopathology 87: 1264–1271

Schell M.A. 2000. Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory network. Annual Review of Phytopathology. 38: 263–292.

Tamura N., Murata Y., and Mukaihara T. 2005. Isolation of *Ralstonia solanacearum* hrpB constitutive mutants and secretion analysis of hrpB-regulated gene products that share homology with known type III effectors and enzymes. Microbiology 151, 2873–2884.

Tans-Kersten J., Huang H., and Allen C. 2001. *Ralstonia solanacearum* needs motility for invasive virulence on tomato. J. Bacteriol. 183:3597–3665.

Timms-Wilson T.M., Bryant K., Bailey M.J. 2001. Strain characterization and 16S–23S probe development for differentiating geographically dispersed isolates of the phytopathogen *Ralstonia solanacearum*. Environ. Microbiol. 3:785–797.

Salanoubat M., Genin S., Artiguenave F., Gouzy J., Mangenot S., Arlat M., Billault A., Brottier P., Camus J.C., Cattolico L., Chandler M., Choisne N., Claudel-Renard C., Cunnac S., Demange N., Gaspin C., Lavie M., Moisan A., Robert C., Saurin W., Schiex T., Siguier P., Thébault P., Whalen M., Wincker P., Levy M., Weissenbach J., and Boucher C.A. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum.* Nature 415:497-502.

Stevens P. and van Elsas J.D. 2010. A putative genomic island, PGI-1, in *Ralstonia solanacearum* biovar 2 revealed by subtractive hybridization. Antonie Van Leeuwenhoek 98(3):359-77.

Stevens P., van Overbeek L.S., and J.D. van Elsas. 2010. *Ralstonia solanacearum* ΔPGI-1 strain KZR-5 is affected in growth, response to cold stress and invasion of tomato. Microb. Ecol. DOI: 10.1007/s00248-010-9728-0.

Vailleau F., Sartorel E., Jardinaud M.F., Chardon F., Genin S., Huguet T., Gentzbittel L., and Petitprez. M. 2007. Characterization of the interaction between the bacterial wilt pathogen *Ralstonia solanacearum* and the model legume plant *Medicago truncatula*. Mol. Plant-Microbe. Interact. 20:159–167.

Van der Wolf J.M., Bonants P.J.M., Smith J.J., Hagenaar M., Nijhuis E., van Beckhoven J.R.C.M., Saddler G.S., Trigalet A., and Feuillade R. 1998. Genetic diversity of *Ralstonia solancaerum* Race 3 in Western Europe determined by AFLP, RC-PFGE and Rep-PCR. Pages 44-49 in: Bacterial Wilt disease: Molecular and Ecological Aspects. P. Prior, C. Allen, and J. Elphinstone, eds. Springer-Verlag, New York.

Van Elsas J.D., Kastelein P., de Vries P.M., and Van Overbeek L.S. 2001. Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* bv. 2 in irrigation water. Can. J. Microbiol. 47(9):842-54.

Vasse J. 1995. Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum*. Mol. Plant-Microbe. Interact. 8:241–251.

Wang L., Makino S., Subedee A., and Bogdanove A.J. 2007. Novel candidate virulence factors in rice pathogen *Xanthomonas oryzae* pv. *oryzicola* as revealed by mutational analysis. Appl. Environ. Microbiol. 73:8023-8027.

Winstead N.N. and Kelman A. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology 42:628-634.

CAPÍTULO 4

Identificación de genes específicos de cepas de *Ralstonia solanacearum* pertenecientes al filotipo II, secuevar 1 y su aplicación al desarrollo de nuevos métodos de diagnóstico basados en PCR

Parte del contenido de este capítulo está publicado como:

Guidot A., Elbaz M., Carrere S., Siri M.I., Pianzzola M.J., Prior P. and Boucher C. 2009. Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathology 99:1105–1112.

RESUMEN

Ralstonia solanacearum es el agente responsable de la marchitez bacteriana, enfermedad que afecta a más de 200 especies vegetales. Las cepas de *R. solanacearum* que causan la marchitez bacteriana de la papa pertenecen al filotipo IIB, secuevar 1 (IIB-1). Estas cepas son responsables de importantes pérdidas económicas en el cultivo de papa y también significan un riesgo para la producción de cultivos ornamentales. Dada la importancia de este patógeno es necesario contar con métodos de detección sensibles y específicos. Como resultado de este trabajo se obtuvo una lista de 70 genes y 15 intergenes específicos para cepas de *R. solanacearum* pertenecientes al grupo IIB-1. Esta lista fue generada mediante hibridación genómica comparativa (CGH) sobre un chip de microarrays que representa el pan-genoma de *R. solanacearum,* y fue validada por PCR sobre una colección de cepas que cubre la diversidad genética conocida en *R. solanacearum.* Estas secuencias específicas están organizadas en nueve regiones que cubren desde 2 hasta 29 genes en el genoma IPO1609, y 6 genes aislados distribuidos en el genoma. Entre los 70 genes específicos, 29 (41%) constituyen elementos genéticos móviles y 34 (49%) forman parte de regiones con uso alternativo de codones (ACURs), lo que sugiere que se originaron a partir de genes adquiridos por transferencia genética horizontal. Teniendo en cuenta la inestabilidad genómica de este patógeno, se seleccionó un grupo de genes que no presentan estas características, para su uso como marcadores específicos de detección. Se desarrolló un método de multiplex PCR, mediante la combinación de *primers* específicos para cepas IIB-1 y *primers* universales para todas las cepas de *R. solanacearum*. El objetivo de este ensayo, es proporcionar una herramienta de diagnóstico útil para la detección de todas las cepas de *R. solanacearum* y la identificación de las cepas de IIB-1 en una única reacción de PCR. Se confirmó la especificidad de este ensayo sobre 85 cepas de *R. solanacearum* y se incorporó una etapa de enriquecimiento previo a la amplificación con el fin de mejorar la sensibilidad de detección sobre extractos de tubérculos de papa (BIO-PCR). Los resultados generados en este estudio contribuyen al desarrollo de métodos de detección más eficientes que ayudan al control de este importante patógeno.

CHAPTER 4

Identification of specific genes for *Ralstonia solanacearum* strains belonging to phylotype II, sequevar 1, and their use as PCR targets for strain detection

---

Part of this chapter was published as:

Guidot A., Elbaz M., Carrere S., Siri M.I., Pianzzola M.J., Prior P. and Boucher C. 2009. Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathology 99:1105–1112.

ABSTRACT

Ralstonia solanacearum is the causal agent of bacterial wilt affecting over 200 different plant species. Potato brown rot strains of *R. solanacearum* from phylotype IIB sequevar 1 (IIB-1), are responsible for important economic losses to the potato industry, and threaten ornamental crop production worldwide. Sensitive and specific detection methods are required to control this pathogen. This study provides a list of 70 genes and 15 intergenes specific to the potato brown rot strains of *R. solanacearum* from phylotype IIB-1. This list was identified by comparative genomic hybridization on a spotted microarray reflecting the pan-genome of *R. solanacearum*, and subsequent PCR validation with a panel of strains covering the known genetic diversity in *R. solanacearum*. The brown rot strain specific genes were organized in 9 clusters encompassing 2 to 29 genes within the IPO1609 genome, and six genes distributed along the genome. Among the 70 specific genes, 29 (41%) are parts of mobile genetic elements and 34 (49%) were classified as alternative codon usage regions (ACURs). This suggested that most of these specific genes originate from acquisition of foreign genes through lateral gene transfers. Considering the known instability of the *R. solanacearum* genome, we further evaluate a subset of genes not predicted to be part of mobile genetic elements or ACURs, thereby sustaining their reliability as specific markers for detection. In this work we developed a multiplex PCR assay by combining a set of IIB-1 specific primers with universal primers for all *R. solanacearum* strains. The goal of this assay, designated as Mx-RsIIB1, is to provide a useful diagnostic tool for detection of all *R. solanacearum* strains and specific identification of IIB-1 potato brown rot strains in the same PCR reaction. Specificity evaluation over 85 strains representative of the diversity found in the *R. solanacearum* species complex, confirmed the suitability of these primers for detection of potato brown rot strains belonging to the IIB-1 group. In order to improve the sensitivity of detection in potato tubers, a BIO-PCR assay was adapted, including an enrichment period prior to amplification. The results generated in this study contribute to the development of specific and reliable detection methods to prevent the spread of this important pathogen.

INTRODUCTION

Ralstonia solanacearum is a Gram-negative soil-borne bacterial plant pathogen with thousands of distinct strains in a heterogeneous species complex. The bacterium causes bacterial wilt in over 200 different plant species covering over 50 botanical families, including dicots and monocots, and annual plants as well as trees (Hayward, 1994b; Elphinstone 2005). Economically important crop hosts include: tomato, potato, pepper, tobacco, peanut, ornamentals, banana, plantain, and eucalyptus.

Several studies were conducted to unravel the phenotypic and genotypic diversity within *R. solanacearum*. Strains were first classified into five races according to host range (Buddenhagen et al., 1962; Pegg and Moffett, 1971; He et al., 1983) and five biovars according to oxidization of various disaccharides and hexose alcohols (Hayward, 1964; 1994a). The race and biovar classifications do not correspond to each other, except that race 3 strains which represent the potato brown rot strains are generally equivalent to biovar 2 (Hayward, 1991). A new classification scheme based on phylogenetic analysis of the internal transcribed spacer (ITS) region was recently proposed (Fegan and Prior, 2005; Prior and Fegan 2005). This classification distinguishes four phylotypes. Phylotype I corresponds to the division 1 of Cook and Sequeira (1994) and contains strains belonging to biovars 3, 4 and 5 and to races 1, 4 and 5. Phylotype II corresponds to the division 2 of Cook and Sequeira (1994) and contains strains belonging to biovars 1, 2 and 2T and to races 1, 2 and 3. Phylotype III contains strains from Africa and the Indian Ocean belonging to biovars 1, 2 and 2T and no race was attributed to this group of strains. Phylotype IV is reported to be highly heterogeneous; it contains strains from Indonesia, Japan, and Australia belonging to biovars 1, 2 and 2T and to races 1 and 3. Phylotype IV also contains the closely related species *Ralstonia syzigii* and the blood disease bacterium (BDB). Each phylotype can be further subdivided into sequevars based on differences in partial sequence of the endoglucanase gene (*egl*). This phylotype classification is broadly consistent with the former race/biovar classification and in some cases gives an indication of the geographical origin and/or the pathogenicity of the strains. It is believed that, after the race/biovar classification, the phylotype classification is to become the core organizing

principle for assigning a particular strain a phylogenetic position with a predictive value on potential host range (Fegan et al., 1998; Wicker et al., 2007).

Whole genome sequencing of a broad host range tomato phylotype I strain (GMI1000) was decisive in unravelling the broad genetic diversity encompassed within *R. solanacearum* (Salanoubat et al., 2002). A microarray representing the GMI1000 genes was developed (Occhialini et al., 2005) and used for comparative genomic hybridizations between 18 strains. Interestingly, this demonstrated that a third of the *R. solanacearum* genome is constituted of variable genes probably acquired by horizontal gene transfers (Guidot et al., 2007). The distribution of variable genes between strains is related to the phylotype classification (Guidot et al., 2007).

Recent phylogenetic evidences indicated that strains that fit with the definition of the potato brown rot agent were placed into the phylotype IIB sequevar 1, *i.e*. the biovar 2 Andean strains of *R. solanacearum* historically known as race 3 biovar 2. These strains are highly pathogenic to potato and tomato, and adapted to highland temperatures. Strains that clustered into three of the four phylotypes of *R. solanacearum* can wilt potatoes, but it is the phylotype IIB sequevar 1 strains (IIB-1 strains) that are the most persistent and potentially the most destructive for potatoes. These IIB-1 strains are specifically dangerous because they can cause symptomless latent infection in seed potato tubers (Ciampi et al., 1980) but also in geranium (Janse et al., 2004; Kim et al., 2003; Sanchez Perez et al., 2008; Swanson et al., 2007) and be disseminated worldwide through imported cuttings or nurseries.

In the European Union (EU) legislation, *R. solanacearum* was listed as a major quarantine organism to control and eradicate, and the potato brown rot strains of *R. solanacearum* were placed in the USA Bioterrorism Select Agent list (Lambert, 2002; USDA, 2004). Given the important economic impact of bacterial wilt, in addition to the potential threat of this bacterium worldwide, it is highly desirable to develop methods for specifically detecting strains of *R. solanacearum*. Such methods, however, need to consider the high phenotypic and genotypic diversity of *R. solanacearum* strains. Several papers relate to methods for specific detection of potato brown rot strains of *R. solanacearum* (Fegan et al., 1998; Griep et al., 1998; Weller et al., 2000). Serological techniques were developed (Griep et al., 1998; Priou et al., 1999), however, cross-

reactions with saprophytic bacteria from potato and soil may give false-positive results. Molecular techniques based on PCR assays appeared more promising. However, all the PCR methods described so far were based on the amplification of the potato-brown-rot-strain specific DNA sequence identified by Fegan et al. (1998) (Weller et al., 2000; patent WO 2004/042016 A2). This sequence encodes a mobile genetic element, i.e. a protein homologous to the Mu-like phage of the ORF35 of the B3 bacteriophage from *Pseudomonas aeruginosa* (Accession Q7AX27). To ensure specificity and reliability of the detection of a pathogenic microorganism, targeting of mobile genetic elements should, however, be avoided since particular strains of the pathogenic microorganism may lose the element or, conversely, other unrelated microorganisms may harbour the element, thereby yielding respectively false negative and false positive results.

Recently, DNA microarrays were reported to be powerful tools for identification and detection of microorganisms given that thousands of probes can be used simultaneously. This technology has already been used for identification and detection of plant pathogenic microbes (Afshar et al., 2008; Fessehaie et al., 2003; Tambong et al., 2006) and Aittamaa et al. (2008) used it to distinguish several bacterial species pathogenic on potato. *R. solanacearum* however was not included in their study. Considering the high genomic plasticity of *R. solanacearum*, specifically its ability to exchange genetic materials, using only one probe for identification and detection of different pathotypes is not adapted because the targeted probe could potentially move from one pathotype to another. The use of DNA microarrays with thousands of probes should strengthen the reliability of the detection method. For such purpose, the list of specific probes for each *R. solanacearum* pathotype must be established.

Lists of genes specific to two IIB-1 strains, UW551 and IVIA 1602, were established by comparative genomic analysis between the completely sequenced genome of phylotype I strain GMI1000 against genome sequences of UW551 and IVIA 1602 (Gabriel et al., 2006; Terol et al., 2006). A total of 402 and 48 genes were detected to be present in strains UW551 and IVIA 1602 respectively and absent in strain GMI1000. The low number of specific genes obtained for the last strain is explained by the low percentage (6.38%) of genome sequenced (Terol et al., 2006). However, the specificity

of these genes in other IIB-1 strains was not validated except for one region detected in strain UW551 where set of 38 contiguous genes (RRSL02400-RRSL02437) was present in all 20 IIB-1 strains tested and absent in other 36 non IIB-1 strains (Gabriel et al., 2006).

In the present work, we established the list of genes that are specific to potato brown rot IIB-1 strains of *R. solanacearum*. The list was established by comparative genomic hybridization (CGH) on a microarray representing the pan-genome of *R. solanacearum* and subsequently validated by PCR amplification. Based on the analysis of this list, a set of genes was selected and evaluated as targets for specific detection of phylotype IIB-1 strains. Finally, a multiplex PCR assay was developed by combining a set of specific IIB-1 primers with universal primers for all *R. solanacearum* strains (Opina et al., 1997). The application of this assay for detection of *R. solanacearum* in potato seed extracts was evaluated.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 4-1. Nine IIB-1 *R. solanacearum* strains isolated from potato in Uruguay were described in Chapter 2. In this study we also included a strain isolated from tomato identified as phylotype IIA, sequevar 5. 61 *R. solanacearum* strains from different origins and comprising all phylotypes were provided by C. Boucher (LIPM-INRA, Toulouse) and E. Verdier (Governmental Plant Protection Service, Montevideo, Uruguay). Other bacterial species used to determine specificity of PCR assays, included 10 strains isolated from different environmental samples in Uruguay.

R. solanacearum strains were routinely cultured at 28ºC either in casamino acid peptone glucose (CPG) (Hendrick and Sequiera, 1984) or tetrazolium chloride agar (TZC) (Kelman, 1954). For the BIO-multiplex PCR assay, *R. solanacearum* was enriched on modified SMSA broth (mSMSA) (Elphinstone et al., 1996).

Identification of candidate genes specific for IIB-1 *R. solanacearum* strains

The list of candidate IIB1-specific genes was deduced from comparative genomic hybridization (CGH) analysis on a microarray chip generated by C. Boucher and collaborators (INRA-CNRS, Toulouse, France). This spotted microarray consisted of 6515 oligonucleotides representative of the genes identified from the genomes of 3 *R. solanacearum* strains belonging to different phylotypes or sequevars: GMI1000 (I-18), IPO1609 (IIB-1), and Molk2 (IIB-3). Genomic DNA extraction and labeling, microarray hybridization, and data analysis were described in Chapter 2. 32 *R. solanacearum* strains were analyzed by CGH, including 10 Uruguayan *R. solanacearum* strains and 22 strains with different origins and phylotypes (Table 4-1). CGH experiments for these latter strains were performed by A. Guidot and M. Elbaz (LIPM-INRA, Toulouse, France). The presence or absence of each oligonucleotide spotted in the microarray chip was established for all tested strains as described in Chapter 2, and candidate IIB-1 specific genes were identified as present in at least 11 of 12 IIB-1 *R. solanacearum* strains and absent in at least 19 of 20 non IIB-1 strains (Table 4-2).

Table 4-1. List of *Ralstonia solanacearum* strains and other bacteria used in this study

***R. solanacearum* strains**						
Strain ID	Host	Origin	Phylotype-Sequevar	Biovar	Analysis [a]	Source [b]
UY031	Potato	Uruguay	IIB-1	2	A, B, C	1
UY032	Potato	Uruguay	IIB-1	2	A	1
UY033	Potato	Uruguay	IIB-1	2	A, B	1
UY034	Potato	Uruguay	IIB-1	2	A	1
UY035	Potato	Uruguay	IIB-1	2	A	1
UY036	Potato	Uruguay	IIB-1	2	A, B, C	1
UY041	Potato	Uruguay	IIB-1	2	A, C	1
UY042	Potato	Uruguay	IIB-1	2	A, B	1
UY043	Soil	Uruguay	IIB-1	2	A, C	1
UY084	Potato	Uruguay	IIB-1	2	C	1
UY094	Potato	Uruguay	II-ND	2	C	1
IPO1609	Potato	Netherlands	IIB-1	2	A, B, C	2
JT516	Potato	Reunion Is.	IIB-1	2	A, B, C	2
CMR34	Tomato	Cameroon	IIB-1	2	A, B, C	2
PSS525	Potato	Taiwan	IIB-1	2	B	2
CMR24	Potato	Cameroon	IIB-1	2	B	2
UW551	Geranium	Kenya	IIB-1	2	C	2
Molk2	Banana	Philippines	IIB-3	1	A, C	2
CIP418	Peanut	Indonesia	IIB-3	1	A, C	2
UW9	Heliconia	Costa Rica	IIB-3	1	B	2
CFBP1183	Heliconia	Costa Rica	IIB-3	1	B	2
UW163	Plantain	Peru	IIB-4	1	A	2
Ant75	Heliconia	Martinique	IIB-4	1	A, C	2
Ant80	Anthurium	Martinique	IIB-4	1	A	2
Ant307	Anthurium	Martinique	IIB-4	1	A, C	2
JY200	Anthurium	Martinique	IIB-4	1	A	2
JY201	Anthurium	Martinique	IIB-4	1	A	2
Ant112	Anthurium	Martinique	IIB-4	1	A, B	2
CFBP6797	Solanum	Martinique	IIB-4	1	B	2
ISBSF1503	Cucumis	Brazil	IIB-4	ND	C	2
CFBP7014	Anthurium	Trinidad	IIB-4	1	B, C	2
CIP10	Potato	Peru	IIB-2T	2T	B	2
NCPPB3987	Potato	Brazil	IIB-2T	2T	B, C	2
ISBSF1712	Geranium	Brazil	IIB-2T	2T	C	2
UY051	Tomato	Uruguay	IIA-5	1	A, B, C	3
A3909	Heliconia	Hawaii	IIA-6	1	A, B, C	2
ICMP7963	Potato	Kenya	IIA-7	1	B, C	2
B34	Banana	Brazil	IIA-24	1	A, B, C	2
CFBP2957	Tomato	Martinique	IIA-36	1	B, C	2
CIP239	Potato	Brazil	IIA-ND	1	B, C	2
CIP301	Potato	Peru	IIA-ND	1	B, C	2
CMR39	Tomato	Cameroon	IIA-ND	1	B, C	2
UW21	Banana	Honduras	IIA-ND	ND	C	2
ISBSF1900	Banana	Brazil	IIA-ND	ND	C	2
JT525	Geranium	Reunion Is.	III-19	1	B, C	2
JT528	Potato	Reunion Is.	III-19	1	B	2

Table 4-1. Continue

Strain ID	Host	Origin	Phylotype-Sequevar	Biovar	Analysis [a]	Source [b]
CMR15	Tomato	Cameroon	III-29	2T	A, C	2
CMR32	Solanum	Cameroon	III-ND	2T	A, B, C	2
CMR43	Potato	Cameroon	III-ND	2T	B, C	2
CMR66	Solanum	Cameroon	III-ND	2T	B, C	2
CIP358	Potato	Cameroon	III-ND	2T	B, C	2
J25	Potato	Kenya	III-ND	2T	B, C	2
CFBP3059	Eggplant	BurkinaFaso	III-ND	1	A, B, C	2
NCPPB332	Potato	Zimbabwe	III-ND	1	B, C	2
NCPPB342	Tabaco	Zimbabwe	III-ND	ND	C	2
NCPPB1029	Geranium	Reunion Is.	III-ND	ND	C	2
GMI1000	Tomato	Guyana	I-18	3	A, C	2
CMR134	Solanum	Cameroon	I-ND	3	A, C	2
CIP365	Potato	Philippines	I-ND	3	B, C	2
PSS81	Tomato	Taiwan	I-ND	3	C	2
PSS190	Tomato	Taiwan	I-ND	3	A, C	2
PSS219	Tomato	Taiwan	I-ND	3	B, C	2
PSS358	Tomato	Taiwan	I-ND	3	A, C	2
R288	Morus	China	I-ND	5	B, C	2
Psi07	Tomato	Indonesia	IV-10	2T	B, C	2
Psi36	Tomato	Indonesia	IV-ND	2T	B	2
ACH732	Tomato	Australia	IV-ND	2T	B, C	2
MAFF301558	Potato	Japan	IV-ND	2T	B, C	2
R. syzygii	Clove	Indonesia	IV-ND	-	A, B, C	2
ICMP7985	Tomato	New Zeland	ND	1	C	4
ICMP8105	Potato	New Zeland	ND	1	C	4
DGSA32	Tomato	Brazil	ND	1	C	4
DGSA95	Potato	Brazil	ND	1	C	4

Other bacteria

Strain	Isolation	Origin	Analysis [a]	Source [b]
Erwinia herbicola	Eucalyptus	Uruguay	C	5
Pseudomonas sp.	Eucalyptus	Uruguay	C	5
Pseudomonas sp.	Carrot	Uruguay	C	5
Rhodomonas sp.	Eucalyptus	Uruguay	C	5
Azospira sp.	Water	Uruguay	C	6
Acidovorax sp.	Water	Uruguay	C	6
Zooglea sp.	Water	Uruguay	C	6
Pseudomonas sp.	Water	Uruguay	C	6
Acinetobacter sp.	Water	Uruguay	C	6
Achromobacter sp.	Water	Uruguay	C	6

ND: not determined.

[a] A: Comparative Genomic Hybridization; B: PCR validation of candidate IIB-1 specific genes; C: Specificity evaluation of PCR assays for detection of IIB-1 *R. solanacearum* strains.

[b] 1: *R. solanacearum* strains isolated from potato fields in Uruguay as described in Chapter 2; 2: *R. solanacearum* strains provided by C. Boucher (LIPM-INRA, Toulouse); 3: *R. solanacearum* strain isolated from tomato in Uruguay; 4: *R. solanacearum* strains provided by E. Verdier (Plant Protection Service, Montevideo); 5: Entophytic strains isolated by P. Rodriguez (DEPBIO-Facultad de Química, Montevideo); 6: Environmental strains isolated from water samples from Raigón aquifer in Uruguay by I. Bellini (DEPBIO-Facultad de Química, Montevideo).

PCR validation

The list of candidate IIB-1 specific genes was validated by PCR amplification of 9 IIB-1 *R. solanacearum* strains and 32 strains representing others phylotypes and sequevars of the *R. solanacearum* species complex (Table 4-1). The PCR primers used (Table 4-3) were designed to amplify one genomic fragment from each gene based on the sequence of strain IPO1609. When possible, one of the 2 primers for each gene was designed within the sequence of the oligonucleotide spotted on the microarray. PCRs were conducted in 25 µl reaction mixture containing 10 ng DNA from each tested strain, 25 pmol of each primer (L/R), 1.5 mM $MgCl_2$, 0.2 mM of each four dNTP, 0.5 U of Red Gold Star *Taq* DNA polymerase (Eurogentec) and the buffer supplied by the manufacturer. PCR amplifications were performed as follows: an initial denaturation step at 96°C for 5 min followed by 30 cycles of 94°C for 15 s, 59°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 10 min. Negative (PCR reaction mixture without DNA) and positive (IPO1609 DNA) controls were included in each experiment. The multiplex PCR described by Fegan and Prior (2005) for the *R. solanacearum* phylotype identification was conducted on each tested DNA as a positive amplification control. PCR products (8 µl) were analyzed by electrophoresis through 1.2 % (w/v) agarose gels with 0.5 µg/ml ethidium bromide and photographed under UV light.

Selection of target genes, primer design, and PCR reactions for specific detection of IIB-1 strains

A subset of IIB-1 specific genes was selected as specific targets for detection of IIB-1 *R. solanacearum* strains (Table 4-4). For these genes, additional primers were designed to amplify PCR products ranging from 300 to 600-bp. When possible, several primer pair combinations were designed in order to cover different regions of each target gene. Primers were designed based on the sequence of strain IPO1609 using Primer 3 Plus (Untergasser et al., 2007) and Vector NTI Advance 11.0 sequence analysis software package (Invitrogen), and were purchased from Macrogen services (Kumchun-ku, Seoul, Korea).

PCRs reactions were conducted in a total volume of 25 µl containing 50 ng of template DNA, 10 pmol of each primer, 1.5 mM $MgCl_2$, 0.2 mM of each four dNTP, 1 U of *Taq*

DNA polymerase (Invitrogen) and the buffer supplied by the manufacturer. PCR amplifications were performed with an initial denaturation step at 96°C for 5 min; followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s; with a final extension step of 72°C for 10 min.

In addition, a multiplex PCR assay was developed based on the utilization of a selected IIB1-specific primer pair in combination with universal *R. solanacearum* primers 759/760 (Opina et al, 1997). Multiplex PCRs were performed in a total volume of 25 µl containing 50 ng of template DNA, 10 pmol of universal primers (759-760), 10 pmol of IIB1-specific primers (02103F1-02103R2), 1.5 mM $MgCl_2$, 0.2 mM of each four dNTP, 1,5 U of *Taq* DNA polymerase (Invitrogen) and the buffer supplied by the manufacturer. PCR amplifications were performed under the same cycling conditions as described above.

Negative controls (PCR reaction mixture without DNA) were included in all reactions. PCR products (8 µl) were analyzed by electrophoresis through 1.5 % (w/v) agarose gels with 0.5 µg/ml ethidium bromide and photographed under UV light.

Specificity of the PCR assays

In order to evaluate the specificity of the primers, a panel of 10 IIB-1 *R. solanacearum* strains and 45 strains representing others phylotypes and sequevars of *R. solanacearum* species complex were tested (Table 4-1). In addition, PCR assays were also performed with other 10 isolates obtained from environmental samples (Table 4-1).

Sensitivity of the PCR assays

To test for DNA sensitivity, genomic DNA from strain UY031 was adjusted to 25 ng/µl with sterile distilled water, and 10-fold serial dilutions were made down to 2,5 pg/µl. Aliquots (2 µl) from each dilution were tested in duplicated with primer pair 02103F1/02103R2 and with multiplex PCR as described above. To determine detection threshold in cell suspensions, bacterial colonies of strain UY031 grown for 48 h on CPG plates were suspended in sterile saline solution and the concentration of the bacterial suspension was spectrophotometrically adjusted to 10^8cfu/ml (optical density at 600

nm of 0.1). A 10-fold dilution series (10^{-1} to 10^{-7}) in sterile distilled water were carried out and 100 µl of dilutions 10^{-4} to 10^{-7} were plated onto TZC medium to confirm actual cell concentration in suspensions. 100 µl of each dilution were boiled for 10 min and aliquots (5 µl) were assayed with the mutiplex PCR assay as described above. All the samples were analyzed in duplicate.

Analysis of inoculated potato tuber extracts

Multiplex PCR reaction was applied for detection of *R. solanacearum* in potato tubers. In order to improve the sensitivity of detection, a BIO-PCR assay was performed, including an enrichment period of 48 h on mSMSA broth prior to amplification.

Potato tuber extracts were prepared using the method described by Ozakman and Schaad (2003) and provided by the governmental Plant Protection Service in Uruguay. Briefly, core tissue was removed aseptically from the stem end of 200 potato tubers and placed into a flask containing 25 mL of 50 mM phosphate buffer, pH 7.2. After shaking for 4 h at room temperature, the suspension was centrifuged at 12, 000 r.p.m. for 10 min at 4°C and suspended in 1 mL of 10 mM phosphate buffer, pH 7.2.

To determine the detection threshold of the BIO-multiplex-PCR assay, strain UY031 was used to prepare spiked potato core tissue extracts. Bacterial suspension was prepared and 10-fold serial diluted with sterile distilled water as described above. 100 µl of the original suspension and each dilution were then added to 900 µl of potato core tissue extracts achieving estimated densities ranging from 10^7 to 1 cfu/ml. To confirm actual cell concentration in suspensions 100 µl of dilutions 10^{-4} to 10^{-7} were plated onto TZC medium and incubated at 28ºC for 48 h. 100 µl aliquots of each spiked potato extract were boiled for 10 min and aliquots (5 µl) were assayed by direct mutiplex PCR assay as described above. At the same time, 100 µl of each dilution in potato extract were added to 900 µl mSMSA broth and incubated for 48 h at 28ºC with shaking at 150 rpm. After this period, 100 µl of each enriched sample were boiled for 10 min and aliquots (5 µl) were assayed by multiplex PCR as indicated above. Non spiked samples were included as negative control, and all the samples were analyzed in duplicate. In addition, 100 µl of each enriched sample was streaked onto mSMSA plates and incubated at 28ºC for 5 days for visual recovery of *R. solanacearum.*

RESULTS

Detection of candidate IPO1609-specific genes among a collection of strains representative of the diversity of *R. solanacearum*

In this study, we used CGH on a spotted microarray reflecting the pan-genome of *R. solanacearum* to compare the gene content of 12 IIB-1 strains and 20 non IIB-1 strains (Table 4-1). This analysis identified a set of 136 oligonucleotides which were detected as present in at least 11 IIB-1 strains and absent in at least 19 non IIB-1 strains (Table 4-2). These oligonucleotides were representative of 77 genes and 38 intergenic regions from the IPO1609 genome. Therefore, these genes and intergenes were considered as "candidate IIB-1-specific genomic regions" (IIB1sgr).

A large proportion of these regions formed clusters in the IPO1609 genome, but a few genes mapping within these clusters were not found in the IIB1sgr list. Based on (i) the known mosaic structure of *R. solanacearum* genome, where complete sets of genes could have been acquired through horizontal gene transfers or lost "en bloc" through deletions (Guidot et al., 2007; 2009; Salanoubat et al., 2002), (ii) the consideration that some genes might be missing from this IIB1sgr list due to lack of hybridization data, and (iii) taking into account the possibility that certain genes that score next to the cut-off value were miscategorized, we included into the IIB1sgr list all the sets of 1 or 2 contiguous genes that were not originally detected as being IIB-1 specific but that are located within a IIB1-specific gene cluster. Based on these criteria, 15 additional genes (identified with a "[a]" in Table 4-3) were included into the list of IIB-1 specific genes. When compared with the sequence of the predicted proteins from strains GMI1000 using BlastP, five of these genes were found to have a counterpart in this strain and were thus eliminated from the final list of IIB-1 specific genes presented in Table 4-3.

Table 4-2. List of oligonucleotides from the *Ralstonia solanacearum* IPO1609 genome detected as present in at least 11 of 12 IIB-1 strains and absent in at least 19 of 20 strains from other phylotypes. The list was established through comparative genomic hybridization on microarray. For each oligonucleotide and strain tested, this table gives the base 2 logarithm of the ratio of the normalized hybridization signal of the tested strain over the normalized hybridization signal of the control DNA (equimolar combination of the genomic DNA from the three strains GMI1000, IPO1609 and Molk2). An oligonucleotide was considered as absent in the tested strain when this value was lower than -1 (in grey).

		IIB-1 strains												non IIB-1 strains																			
Oligo ID	Corresponding Gene ID	IPO1609	JT516	CMR34	UY032	UY042	UY033	UY035	UY036	UY041	UY034	UY043	UY031	GMI1000	CMR14	PSS358	PSS190	CFBP3059	CMR15	CMR32	R. sizygii	UY051	B34	A3909	Molk2	CIP418	UW163	Ant307	Ant80	Ant75	JY201	JY200	Ant112
IPO1609 0648	IPO 00030	1.77	1.59	-2.02	1.40	1.72	2.00	1.50	1.53	1.41	0.76	2.03	1.54	-5.71	-6.81	-9.64	-4.61	-7.10	-4.09	-6.06	-3.47	-3.49	-4.23	-7.98	-6.54	-8.06	-8.92	-5.45	-7.91	-7.31	-5.93	-5.76	-7.47
IPO1609 0647	IPO 00031	1.33	1.41	-7.71	1.55	1.55	1.55	1.66	1.67	0.97	1.31	1.51	1.58	-4.84	ND	-6.69	-2.99	ND	-5.71	-7.20	-5.16	-2.45	-4.43	-1.13	-5.73	-9.73	-9.98	-9.76	-4.45	-8.03	-4.91	-3.81	-3.43
IPO1609 0644	IPO 00043	1.37	1.77	1.52	1.15	1.23	1.69	1.21	1.08	0.96	0.40	1.54	1.24	-5.44	-8.06	-7.82	-5.23	ND	-4.62	-6.75	-6.11	-5.87	-3.82	-8.70	-3.68	-7.64	-6.30	-4.23	-3.89	-4.54	-3.26	-4.23	-2.82
IPO1609 0643	IPO 00044	1.43	1.76	1.87	1.72	1.88	2.01	1.63	1.83	1.41	1.16	2.00	1.73	-4.74	-9.54	ND	-3.91	ND	ND	ND	-5.24	-2.51	-4.46	ND	-7.51	-7.29	-8.85	-8.86	-4.78	-7.03	-5.25	-5.58	-5.86
IPO1609 0641	IPO 00045	1.49	1.84	1.74	1.40	1.54	2.02	1.50	1.43	1.11	0.72	1.83	1.58	-4.68	-8.47	ND	-4.01	-8.82	ND	-7.04	-5.25	-3.66	-3.99	ND	-4.56	-6.33	-7.40	-5.95	-4.61	-4.90	-3.98	-5.14	-3.53
IPO1609 0642	IPO 00045	1.31	1.77	1.55	1.38	1.81	2.15	1.35	1.58	1.41	0.92	1.74	1.38	-5.12	-6.05	-7.67	-4.86	-7.57	-4.64	-7.30	-5.20	-4.57	-5.05	-5.24	-4.70	-6.00	-7.30	-7.00	-5.46	-7.67	-4.65	-4.39	-4.76
IPO1609 0639	IPO 00046/00047	1.46	1.60	1.94	1.71	1.47	1.47	1.86	1.47	1.55	1.33	1.66	1.79	-2.98	ND	ND	-2.93	ND	ND	ND	-2.74	-2.01	2.21	-3.74	-2.67	-6.64	-3.74	-1.39	-1.33	-1.58	-1.35	ND	-0.97
IPO1609 0625	IPO 00232/00233	1.59	1.67	1.33	1.67	2.71	3.16	1.58	2.30	0.97	1.29	2.05	1.16	-5.41	-6.83	ND	-3.03	-6.88	ND	-9.11	-6.01	-5.33	-8.70	-6.86	-5.74	-8.67	-8.20	-7.19	-7.31	-7.50	-5.54	-6.17	-6.64
IPO1609 0626	IPO 00232/00233	1.35	1.66	1.27	1.23	1.39	1.73	1.24	1.59	1.33	1.03	1.49	1.16	-4.59	-5.31	-6.59	-3.34	-5.54	-6.65	-5.63	-4.47	-4.22	-4.91	-4.47	-3.73	-4.49	-6.48	-4.41	-4.83	-4.70	-4.03	-4.29	-4.38
IPO1609 0624	IPO 00233	1.46	1.65	1.23	1.54	1.94	2.54	1.54	1.77	0.99	1.00	1.89	1.47	-4.37	-3.79	-7.18	-2.70	-3.84	-7.43	-5.04	-4.60	-4.15	-5.15	-4.03	-4.23	-5.67	-5.42	-2.97	-4.76	-3.70	-3.86	-4.16	-3.76
IPO1609 0623	IPO 00234	1.21	1.61	1.23	1.29	2.04	2.04	1.29	1.71	0.90	1.14	1.59	1.24	-2.82	-1.98	-2.52	-1.59	-1.82	-2.19	-2.23	-1.28	-2.47	-2.62	-1.98	-2.31	-2.34	-2.46	-1.88	-1.89	-2.08	-2.26	-2.04	-2.31
IPO1609 0267	IPO 00874/00875	1.70	1.35	1.29	1.97	4.13	4.33	2.00	3.04	0.86	0.73	2.15	1.65	-5.63	-8.19	-9.61	-5.71	-4.98	ND	ND	-4.02	-6.40	-5.62	-6.56	-4.69	-3.58	-2.13	-1.93	-2.69	-2.12	-2.52	-2.13	-2.63
IPO1609 0266	IPO 00875	1.30	1.80	1.93	1.35	1.45	1.99	1.57	1.66	1.19	1.13	1.60	1.40	-5.49	ND	-6.40	-4.71	-4.24	-4.29	ND	-5.28	-5.29	-6.43	ND	-5.22	-6.07	-10.21	-6.61	-7.04	-7.46	-5.38	-7.43	-5.36
IPO1609 0265	IPO 00875/00876	1.39	1.82	1.81	1.50	2.49	2.66	1.55	2.12	1.13	1.43	1.96	1.34	-5.77	ND	ND	-5.18	-5.86	ND	ND	-5.93	-4.94	-8.43	ND	-3.18	-3.13	-4.02	-7.82	-5.33	-8.31	-6.00	-5.07	-6.34
IPO1609 0272	IPO 00876	1.33	1.79	2.02	1.55	1.94	2.07	1.65	1.90	0.90	1.07	1.50	1.54	-4.16	-6.94	-7.31	-2.61	-6.62	-4.10	-6.40	-4.18	-3.22	-4.86	ND	-3.90	-6.16	-9.74	-3.69	-4.72	-4.59	-3.27	-3.31	-3.15
IPO1609 0271	IPO 00877	1.44	1.75	1.74	1.35	2.28	2.81	1.64	2.04	1.05	0.49	2.01	1.45	-4.96	-7.48	-7.97	-4.58	-3.71	-6.91	-4.94	-5.73	-2.31	-4.90	-3.84	-2.74	-4.15	-5.05	-2.62	-3.18	-3.06	-2.25	-2.55	-1.94
IPO1609 0270	IPO 00878	1.32	2.34	2.37	1.53	2.02	2.47	1.72	1.71	0.95	0.94	2.05	1.49	-5.23	ND	ND	-3.63	-7.29	-7.61	-7.64	-5.30	-3.34	-6.70	-7.53	-3.95	-4.03	-7.58	-5.68	-5.03	-5.65	-4.29	-4.49	-3.41
IPO1609 0269	IPO 00878/00879	1.42	1.65	1.56	1.35	1.61	2.04	1.55	1.69	0.86	0.90	1.65	1.43	-4.20	-6.12	-6.37	-3.93	-5.43	-9.00	-7.12	-4.84	-4.88	-3.71	-3.91	-2.35	-1.93	-2.31	-1.98	-1.92	-1.93	-2.06	-1.89	-2.06
IPO1609 0313	IPO 00878/00879	1.34	0.47	1.49	1.28	1.95	2.20	1.35	1.87	0.60	0.72	1.09	1.14	-8.23	-7.94	-6.70	-5.10	-5.46	-6.14	-6.94	-8.00	-4.59	-5.22	-1.02	-5.99	-2.86	-3.74	-4.10	-5.10	-5.27	-4.97	-4.92	-4.49
IPO1609 0268	IPO 00879	1.42	2.11	1.89	1.75	3.16	3.89	1.84	2.82	0.86	0.63	2.33	1.47	-4.76	-8.12	-8.62	-4.12	-7.56	-4.95	-6.55	-5.49	-4.18	-5.68	-4.73	-4.13	-5.36	-3.98	-2.99	-4.12	-3.11	-3.87	-3.82	-2.98
IPO1609 0659	IPO 00881	1.50	1.73	1.73	1.81	3.47	3.39	1.71	2.31	0.92	0.97	1.83	1.45	-5.19	ND	-6.28	-4.80	ND	-5.47	ND	-4.34	-4.41	-4.04	ND	-5.94	-7.70	-10.04	-6.94	-5.68	-9.80	-6.42	-5.63	-5.90
IPO1609 0151	IPO 00883/00884	1.26	2.04	2.03	1.31	1.61	1.93	1.43	1.49	0.80	1.03	1.65	1.45	-5.35	-5.53	ND	-4.65	ND	ND	ND	-5.38	-4.82	-5.06	ND	-5.14	-8.16	-8.27	-8.27	-4.20	-5.09	-4.27	-4.60	-3.94
IPO1609 0147	IPO 01025/01026	1.51	1.40	1.43	1.67	2.50	2.53	1.73	1.95	0.95	1.47	1.68	1.59	-5.36	-7.57	-7.47	-5.23	-3.75	-4.87	-3.96	-4.69	-4.62	-4.49	-5.07	-2.85	-2.39	-3.40	-2.72	-3.29	-3.03	-2.73	-3.33	-3.03
IPO1609 0146	IPO 01030	1.57	1.84	1.61	1.45	1.38	1.70	1.65	1.71	1.74	1.50	2.05	1.49	-5.05	-2.97	-5.16	-3.89	-4.49	ND	-4.82	-5.49	-4.09	-3.87	-3.72	-2.16	-2.17	-3.30	-3.01	-2.49	-2.71	-2.30	-2.54	-2.89
IPO1609 0141	IPO 01057/01058	1.70	1.70	1.50	1.52	1.60	1.88	1.55	1.69	1.29	1.47	2.14	1.48	-4.90	-5.80	ND	-2.33	-5.91	-5.38	-7.11	-5.13	-5.19	-6.67	-5.40	-4.41	-6.20	-7.62	-5.14	-5.75	-7.89	-4.74	-5.73	-5.48
IPO1609 0135	IPO 01058	1.69	1.37	1.28	1.75	2.73	2.68	1.80	2.02	1.12	0.99	2.40	1.53	-4.05	-4.49	-7.10	-2.50	-7.37	ND	-4.73	-4.66	-4.49	ND	-6.73	-4.45	-7.20	-9.56	-6.21	-5.05	-7.11	-4.30	-4.46	-4.21
IPO1609 0124	IPO 01130/01131	1.75	1.61	1.49	1.57	2.30	2.38	1.75	2.08	1.55	1.31	1.96	1.72	-4.70	-5.55	-6.23	-4.72	-8.61	-4.16	-6.80	-4.25	-4.62	-6.04	-5.51	-5.24	-7.56	-8.13	-5.81	-7.01	-5.50	-5.24	-5.59	-5.20
IPO1609 0123	IPO 01131	1.75	1.45	1.48	1.42	2.01	2.20	1.64	1.82	1.20	0.61	2.09	1.58	-4.98	-6.92	ND	-3.35	-7.11	ND	-7.66	-5.09	-5.22	-6.08	-7.31	-5.63	-5.85	-8.31	-8.41	-5.33	-5.91	-4.68	-5.50	-4.74
IPO1609 0122	IPO 01132	1.84	1.24	1.27	1.64	2.91	3.06	1.78	2.33	1.12	0.90	2.02	1.53	-5.31	-5.85	-6.47	-3.44	-7.36	-4.18	-5.01	-5.57	-5.57	-4.62	-5.72	-4.98	-5.16	-8.30	-4.74	-5.92	-6.13	-5.13	-7.18	-5.24
IPO1609 0127	IPO 01137/01138	1.76	1.63	1.56	2.00	3.34	3.52	2.07	2.67	1.47	1.35	2.46	1.79	-4.80	-7.93	ND	-3.61	ND	ND	ND	-4.66	-4.40	ND	ND	-5.06	-8.29	-9.72	-6.43	-5.10	-6.51	-6.50	-5.48	-4.42
IPO1609 0129	IPO 01137-1	1.45	1.95	1.93	1.63	1.91	1.92	1.60	1.73	1.49	1.75	1.76	1.72	-5.18	-6.55	-6.79	-3.49	-5.70	-4.94	-6.00	-3.57	-3.87	-6.71	-6.18	-3.82	-4.46	-8.35	-7.94	-5.63	-7.16	-5.74	-5.05	-4.60
PT01265B	IPO 01137-2	1.38	1.05	1.04	1.58	2.66	2.25	1.62	1.80	0.67	0.91	1.41	1.51	-2.07	-1.77	-2.49	-2.06	-2.08	-2.20	-2.22	-4.58	-3.44	-2.58	-2.89	-3.25	-2.97	-2.81	-2.35	-2.60	-2.52	-2.26	-2.51	-2.02
IPO1609 0130	IPO 01214/01215	1.70	1.50	1.30	1.50	2.28	2.52	1.64	1.96	1.30	1.14	1.97	1.52	-4.75	-6.08	-5.11	-3.96	-8.70	-5.79	-6.79	-5.28	-5.43	-3.72	-4.37	-3.45	-3.61	-6.30	-4.96	-4.29	-5.62	-4.04	-5.37	-3.97
IPO1609 0128	IPO 01219/01220	1.35	1.77	1.62	1.39	2.72	2.56	1.50	2.12	1.71	ND	1.99	1.40	-4.24	-5.10	-5.52	-2.25	-5.69	-3.81	-4.60	-4.45	-3.68	-4.19	-3.93	-4.04	-4.53	-7.04	-3.69	-4.92	-3.60	-4.30	-4.79	-3.77
IPO1609 0159	IPO 01258/01259	1.83	1.33	1.07	1.71	2.79	2.92	1.87	2.26	1.04	1.36	1.93	1.69	-5.44	ND	ND	-4.60	ND	-7.55	-8.07	-5.93	-5.04	-6.18	ND	-5.52	-6.74	-9.74	-6.07	-5.47	-7.06	-5.71	-5.98	-5.03
IPO1609 0160	IPO 01258/01259	1.84	1.08	1.13	1.64	2.15	2.29	1.89	1.82	1.14	1.29	1.98	1.80	-5.15	-5.05	-7.52	-2.41	-7.53	-3.62	-2.82	-5.66	-4.53	-1.85	-10.24	-4.58	-2.50	-3.37	-2.41	-5.32	-2.92	-5.08	-4.84	-4.00
IPO1609 0161	IPO 01259	1.68	1.60	1.37	1.69	2.24	2.37	1.70	1.95	1.28	1.47	1.81	1.70	-4.10	-7.58	-4.73	-4.47	ND	ND	-6.54	-5.01	-3.05	-5.04	-8.39	-4.55	-7.18	-9.75	-4.75	-5.54	-6.36	-4.81	-4.56	-3.95
IPO1609 0386	IPO 01260	1.31	2.05	1.58	1.50	1.38	1.38	1.78	1.58	1.31	1.16	1.50	1.74	-4.62	-6.72	-6.66	-4.58	-6.26	-4.79	ND	-5.00	1.46	-3.63	ND	-4.92	-7.99	-8.90	-4.87	-8.90	-8.36	-5.84	-6.94	-4.63
IPO1609 0167	IPO 01311	1.41	2.10	1.73	1.52	1.81	2.55	1.61	1.54	1.61	1.45	1.60	1.53	-3.73	-6.19	-2.74	-2.01	-7.40	-7.22	-5.63	-4.27	-2.86	-2.85	-3.06	-3.37	-2.79	-3.98	-2.82	-3.67	-2.97	-2.92	-3.07	-3.11
IPO1609 0168	IPO 01312	1.60	1.88	1.88	1.80	2.02	3.22	1.86	1.85	1.58	1.37	1.92	1.98	-4.73	-7.09	ND	-3.60	-5.88	ND	ND	-4.33	-4.43	-6.46	-6.25	-4.62	-6.29	-8.25	-6.22	-4.39	-6.39	-3.70	-3.20	-3.71
IPO1609 0170	IPO 01314	1.56	1.28	1.41	1.62	1.31	1.99	1.69	1.38	1.52	1.70	1.28	1.54	-3.48	-4.88	-5.37	-2.98	-4.25	-3.68	-5.29	-3.28	-2.23	-2.34	-6.62	-1.75	-3.19	-4.84	-3.81	-2.31	-3.91	-1.81	-2.10	-1.65
IPO1609 0185	IPO 01362	1.44	1.63	1.25	1.53	1.81	1.87	1.78	1.96	1.08	1.37	1.42	1.44	-4.76	-8.61	-5.69	-3.76	-4.87	-4.31	-5.48	-4.59	-3.60	-3.90	-5.88	-5.51	-9.11	-9.01	-3.35	-4.54	-6.95	-3.77	-4.49	-3.97
IPO1609 0216	IPO 01877/01878	1.34	1.18	1.28	1.18	1.23	1.04	1.65	1.19	1.47	1.23	1.32	1.70	-2.36	-5.54	-5.03	-3.07	ND	ND	-6.17	-2.99	-2.80	-4.43	-6.74	-2.05	-1.04	-1.65	-1.40	-3.15	-1.22	-2.60	-4.16	-2.51
IPO1609 0217	IPO 01878/01879	1.29	1.64	1.50	1.34	2.51	2.61	1.37	2.06	0.99	0.43	1.58	1.22	-6.66	-8.23	-8.08	-5.18	-8.44	-4.12	-8.59	-6.09	-2.76	-2.88	-3.43	-2.49	-1.98	-3.89	-2.95	-3.28	-3.76	-3.60	-3.54	-2.98
IPO1609 0218	IPO 01878/01879	1.31	1.57	1.56	1.26	0.77	0.64	1.36	1.03	1.25	0.91	1.39	1.46	-3.91	ND	ND	-3.40	-4.77	-2.55	-10.00	-4.81	-1.41	-1.78	-4.22	-1.11	-1.54	-1.76	-1.33	-1.21	-1.64	-0.94	-1.03	-0.60
IPO1609 0233	IPO 02072/02073	1.18	1.55	1.27	1.21	1.42	1.68	1.33	1.51	0.89	0.92	1.15	1.12	-4.81	-8.04	-5.48	-2.70	-5.41	-3.98	-5.03	-4.53	-1.95	-2.88	-3.20	-2.15	-1.48	-1.69	-1.55	-1.44	-1.70	-1.74	-1.76	-1.76
IPO1609 0234	IPO 02072/02073	1.59	1.58	1.46	1.52	2.43	2.57	1.76	1.90	0.78	0.97	1.74	1.42	-5.49	-9.23	-6.26	-4.73	-4.95	ND	-5.27	-1.44	-3.88	-3.30	-4.69	-3.80	-5.96	-7.77	-3.46	-6.01	-4.75	-4.27	-4.94	-4.75
IPO1609 0244	IPO 02090	1.42	1.58	1.41	1.27	1.32	1.72	1.37	1.56	0.97	0.69	1.40	1.10	-4.92	-6.65	ND	-4.75	-8.24	ND	-3.43	-4.25	-4.96	-5.83	-6.84	-2.33	-1.87	-9.89	-8.89	-6.48	-9.15	-4.36	-4.99	-5.08
IPO1609 0246	IPO 02092	1.59	1.10	1.39	2.01	1.78	1.93	1.70	2.09	1.33	1.19	1.69	1.68	-3.67	-5.99	-6.24	-4.26	-5.62	-0.43	-7.43	-3.87	-4.17	-4.02	-6.88	-1.76	-1.78	-6.95	-6.08	-4.01	-4.63	-3.81	-3.29	-3.44
IPO1609 0247	IPO 02092/02093	1.33	1.68	1.70	2.03	1.46	1.47	1.91	1.86	1.26	1.49	1.66	1.80	-3.21	-5.35	-4.77	-1.54	-6.18	ND	-7.27	-2.63	-3.66	1.26	ND	-2.77	-4.83	-8.71	-5.82	-4.71	-8.27	-3.89	-4.54	-3.40
IPO1609 0249	IPO 02093/02094	0.99	1.58	1.62	1.70	1.27	1.31	1.65	1.64	1.17	1.32	1.35	1.41	-4.74	-5.71	-1.00	-4.39	-6.37	-0.10	-7.42	-4.12	-2.96	-3.46	-3.55	-1.87	-1.82	-6.35	-3.62	-2.98	-4.24	-2.89	-2.75	-2.43
IPO1609 0251	IPO 02095	1.08	1.15	1.18	1.56	1.91	2.04	1.48	1.99	0.85	1.17	1.45	1.19	-4.55	-6.91	-1.01	-4.01	ND	-3.31	ND	-4.85	-4.55	-5.12	-6.36	-1.32	-1.53	-10.53	-6.90	-4.70	-7.43	-4.77	-6.59	-4.94
IPO1609 0252	IPO 02097	1.32	2.16	2.28	1.90	1.46	1.74	1.69	1.93	1.44	0.91	1.79	1.84	-4.20	-5.78	-1.16	-3.91	-5.81	0.34	-5.50	-4.48	-4.00	-5.22	-6.75	-4.10	-8.06	-9.00	-8.28	-6.78	-8.67	-4.80	-6.12	-5.14
IPO1609 0253	IPO 02098	1.34	1.98	1.97	1.89	1.52	1.68	1.78	1.87	1.08	1.07	1.54	1.78	-4.40	-6.88	-5.26	-2.55	ND	1.14	ND	-5.05	-4.83	-6.38	ND	-4.23	-9.84	-9.86	-9.91	-5.34	-9.62	-5.10	-5.74	-4.38
IPO1609 0257	IPO 02100/02101	1.43	2.16	2.24	1.81	2.67	3.04	1.87	2.36	1.50	0.90	1.96	1.72	-4.43	-6.55	-4.80	-4.03	-7.30	-4.00	-5.74	-5.09	-4.15	-5.18	-7.99	-3.65	-6.75	-8.35	-6.89	-4.79	-6.34	-4.08	-4.04	-3.15
IPO1609 0258	IPO 02100/02101	1.53	1.76	1.72	1.62	2.46	2.63	1.75	2.07	1.03	0.89	1.94	1.66	-4.65	ND	-3.63	-2.97	-6.15	-3.23	-4.45	-5.62	-4.52	-4.35	ND	-3.83	-5.30	-9.77	-6.62	-5.14	-9.13	-4.77	-6.85	-5.34
IPO1609 0259	IPO 02100/02101	1.35	2.17	2.20	1.38	1.14	1.72	1.50	1.55	1.38	0.81	1.62	1.52	-3.69	ND	-5.29	-2.54	-6.45	ND	ND	-4.50	-3.98	-3.41	-5.07	-3.64	-5.74	-6.07	-5.92	-4.43	-7.95	-3.67	-4.85	-3.67
IPO1609 0060	IPO 02102	1.40	2.03	2.15	1.46	1.69	1.79	1.63	1.61	1.13	1.15	1.50	1.48	-4.60	ND	ND	-5.03	-3.26	-4.34	ND	-5.73	-3.63	-5.12	-7.75	-6.05	-7.92	-10.47	-8.01	-5.71	-8.74	-4.64	-5.10	-5.59
IPO1609 0061	IPO 02103/02104	1.51	1.93	2.03	1.79	1.77	2.39	1.88	1.47	0.79	1.20	1.63	1.87	-4.09	-5.06	-3.12	-3.96	-3.11	-2.89	ND	-4.58	-5.15	-3.46	-4.72	-4.62	-6.14	-8.39	-4.18	-5.69	-5.98	-2.69	-5.26	-3.49
IPO1609 0065	IPO 02140	1.44	1.54	1.38	1.31	0.91	1.22	1.58	1.23	0.96	1.34	1.63	1.54	-8.14	-9.42	-6.99	-7.86	-7.97	ND	ND	-8.26	-4.59	-3.54	-0.47	-7.81	-12.22	-9.02	-9.76	-8.57	-7.93	-8.39	-8.83	-7.22
IPO1609 0066	IPO 02141	1.37	1.59	1.60	1.71	1.14	1.41	1.29	1.45	1.00	0.94	1.37	1.41	-4.86	-10.45	ND	-4.70	-7.05	-5.61	-7.54	-5.02	-5.55	-4.75	ND	-5.78	-8.95	-9.43	-7.66	-7.72	-9.84	-6.49	-7.77	-5.37
IPO1609 0067	IPO 02141/02142	1.68	1.47	1.47	2.02	1.55	1.61	1.83	1.57	1.23	1.36	1.76	1.76	-3.68	ND	ND	-3.85	-7.67	0.27	ND	-4.35	-3.67	-3.59	-3.72	-4.88	-8.44	-8.38	-8.46	-5.59	-7.41	-5.04	-6.74	-5.06
IPO1609 0068	IPO 02142	1.41	1.11	1.24	2.22	1.91	1.61	1.68	1.72	1.05	1.76	1.28	1.65	-2.96	-5.49	-1.79	-1.70	-5.58	-2.08	-5.02	-2.57	-1.87	-3.77	-6.57	-3.33	-6.48	-8.58	-5.82	-3.46	-7.58	-3.09	-2.94	-2.81
IPO1609 0069	IPO 02143	1.29	1.38	1.52	1.72	1.52	1.50	1.28	1.42	0.73	0.89	1.18	1.39	-4.50	-5.31	-7.28	-4.46	-5.22	2.18	-5.70	-4.22	-3.93	-3.74	-4.49	-4.59	-3.82	-4.22	-2.72	-3.29	-3.40	-3.63	-3.02	-2.92
IPO1609 0070	IPO 02144	1.71	1.41	1.60	2.20	2.86	2.79	1.73	2.14	1.06	1.53	1.96	1.68	-4.64	ND	ND	-3.54	ND	0.94	-7.20	-4.61	-4.47	-6.43	ND	-5.65	-7.12	-9.07	-7.36	-6.60	-7.08	-5.66	-4.46	-4.66
IPO1609 0071	IPO 02145	1.45	1.73	1.80	2.14	1.47	1.08	1.83	1.36	1.39	1.41	1.25	1.95	-3.07	-6.42	-7.15	-3.12	-4.49	-5.57	ND	-3.46	-2.67	-4.27	-4.64	-2.70	-2.32	-5.70	-2.76	-3.12	-3.28	-2.43	-2.77	-2.13
IPO1609 0072	IPO 02146	1.30	0.55	1.36	1.39	0.85	0.87	1.25	0.11	0.65	1.28	1.16	1.39	-3.04	-5.20	-2.85	-1.27	-5.12	-1.58	-4.57	-3.26	-2.50	-3.79	-4.58	-3.91	-4.21	-5.38	-4.79	-3.71	-4.77	-2.72	-3.50	-3.40
IPO1609 0074	IPO 02147	1.52	1.84	1.91	2.04	1.41	1.45	1.55	1.39	1.58	1.09	1.61	1.62	-3.41	ND	ND	-2.08	ND	-5.61	-6.75	-3.84	-3.04	-3.32	ND	-3.61	-7.99	-8.66	-5.85	-4.99	-8.47	-4.64	-4.90	-3.25
IPO1609 0076	IPO 02148	1.05	0.95	0.74	1.03	1.63	1.57	1.15	1.15	0.49	0.66	1.02	1.07	-2.40	-2.11	-1.40	-1.51	-1.85	0.85	-1.11	-4.76	-1.77	-3.35	-4.93	-4.86	-4.42	-4.97	-3.59	-3.73	-5.09	-4.10	-4.48	-3.87
IPO1609 0077	IPO 02149	1.63	1.23	1.42	2.14	1.37	1.64	1.63	1.55	1.22	1.01	1.69	1.51	-4.04	-7.21	-6.53	-3.97	ND	0.89	-7.10	-4.15	-3.76	-2.38	-2.53	-3.69	-1.42	-1.78	-1.83	-4.97	-1.98	-4.79	-4.04	-3.86
IPO1609 0078	IPO 02150	1.58	1.78	1.92	2.47	2.23	2.16	2.02	2.02	1.45	1.61	1.74	1.97	-2.76	ND	-7.17	-1.63	ND	-1.48	-6.18	-3.57	-3.05	-3.19	ND	-3.85	-6.44	-8.17	-7.13	-5.60	-7.28	-2.86	-2.71	-3.01

Table 4-2. Continue

		IIB-1 strains												non IIB-1 strains																		
Oligo ID	Corresponding Gene ID	IPO1609	JT516	CMR34	UY032	UY042	UY033	UY035	UY036	UY041	UY034	UY043	UY031	GMI1000	CMR14	PSS358	PSS190	CFBP3059	CMR15	CMR32	R. sizygii	UY051	B34	A3909	Molk2	CIP418	UW163	Ant307	Ant80	Ant75	JY201	JY200	Ant112
IPO1609_0080	IPO_02151	1.65	1.78	1.48	2.13	2.46	2.20	1.69	1.56	1.35	1.19	1.83	1.65	-4.57	ND	ND	-2.78	ND	-5.98	-6.97	-4.89	-4.80	-5.53	ND	-6.15	-8.75	-9.98	-6.48	-5.48	-9.46	-5.90	-4.12	-5.08
IPO1609_0081	IPO_02152	1.15	2.20	1.91	1.93	1.17	1.11	1.55	1.42	1.54	1.02	1.54	1.58	-4.72	-5.73	-5.57	-3.14	-7.33	-4.42	ND	-4.23	-3.74	-3.81	ND	-4.67	-5.84	-6.25	-5.97	-4.86	-4.61	-3.96	-4.06	-3.50
IPO1609_0082	IPO_02153	1.02	1.82	1.77	1.74	1.24	1.20	1.29	1.22	1.26	1.14	1.22	1.41	-4.03	ND	-3.41	-2.48	-5.50	-5.83	-6.93	-3.31	-3.06	-3.62	-6.02	-4.53	-6.97	-8.43	-5.49	-5.24	-7.58	-4.12	-5.07	-4.70
IPO1609_0083	IPO_02154	1.47	1.40	1.27	1.88	1.70	1.65	1.61	1.32	1.23	1.00	1.66	1.64	-7.27	-5.70	-7.83	-3.02	-6.86	-8.29	-3.74	-6.74	-1.62	-1.20	-5.14	-1.97	-1.14	-1.46	-1.13	-2.85	-1.23	-1.49	-1.98	-1.56
IPO1609_0084	IPO_02154/02155	1.42	1.84	1.79	2.14	1.42	1.21	1.70	1.52	1.66	1.57	1.44	1.72	-3.56	-6.36	ND	-3.72	-9.82	-3.18	-6.81	-3.85	-3.20	-4.74	-7.60	-4.32	-4.91	-8.37	-5.14	-4.76	-8.28	-4.44	-4.14	-4.03
IPO1609_0085	IPO_02155	1.44	1.51	1.55	1.88	1.71	1.69	1.54	1.37	1.41	1.09	1.48	1.59	-4.69	-6.19	-6.96	-4.10	-8.37	-3.17	ND	-4.43	-3.12	-4.27	-8.26	-2.56	-3.67	-6.61	-4.31	-4.54	-4.40	-3.34	-3.93	-3.51
IPO1609_0086	IPO_02156	1.37	1.65	1.75	2.00	2.46	1.44	1.76	1.60	1.28	1.73	1.44	1.76	-3.09	-5.17	-6.36	-1.76	-5.38	-4.33	-6.81	-0.51	-2.74	-4.84	ND	-3.13	-3.16	-5.25	-3.11	-2.12	-4.45	-1.77	-1.80	-1.70
IPO1609_0087	IPO_02157	1.44	1.88	1.83	2.01	1.63	1.65	1.52	1.61	1.45	0.81	1.53	1.47	-4.63	-5.97	-7.81	-4.73	-6.88	1.71	ND	-5.08	-4.63	-4.42	-7.34	-5.18	-7.41	-9.33	-6.42	-5.01	-8.20	-8.29	-4.58	-4.35
IPO1609_0088	IPO_02158	1.75	1.48	1.42	2.27	2.40	2.34	1.72	1.95	1.23	1.46	1.83	1.68	-4.15	-5.79	-4.88	-3.16	-7.31	-0.04	-7.53	-4.86	-3.54	-3.87	-6.60	-4.93	-8.75	-9.61	-6.24	-4.56	-7.10	-3.47	-4.36	-3.60
IPO1609_0089	IPO_02159	1.45	1.54	1.47	1.84	1.83	2.00	1.62	1.62	1.15	1.05	1.41	1.10	-4.20	-6.68	ND	-3.32	-5.97	0.33	ND	-4.72	-3.98	-5.48	-6.01	-4.10	-9.87	-9.78	-7.22	-4.52	-8.12	-3.58	-4.91	-4.64
IPO1609_0090	IPO_02159/02160	1.63	1.70	1.62	2.30	2.72	2.56	1.91	2.12	1.41	1.74	1.75	1.21	-4.16	-6.19	-7.83	-2.71	-9.57	ND	-7.30	-4.41	-3.73	-5.92	-8.65	-4.80	-9.59	-9.19	-7.64	-5.71	-8.52	-3.93	-5.18	-4.49
IPO1609_0091	IPO_02159/02160	1.29	1.68	1.96	2.24	2.01	2.10	1.92	1.90	1.12	1.39	1.39	1.40	-3.11	-6.06	ND	-2.95	ND	0.62	ND	-3.07	-2.70	-4.81	ND	-1.44	-1.83	-6.28	-4.53	-2.24	-4.38	-1.45	-1.98	-1.83
IPO1609_0092	IPO_02160	1.27	1.95	1.91	1.76	1.54	1.53	1.32	1.53	1.23	1.19	1.28	0.92	-3.83	-4.74	-6.36	-2.73	-5.83	1.73	-5.56	-4.27	-2.54	-4.09	-4.94	-3.23	-3.35	-6.22	-3.35	-3.59	-4.18	-3.02	-3.63	-3.64
IPO1609_0093	IPO_02162	1.59	1.30	1.27	2.10	2.19	2.30	1.55	1.97	0.99	1.35	1.46	1.49	-4.30	-6.49	-6.80	-4.42	-6.85	1.23	-8.74	-2.14	-2.38	-2.27	-2.91	-2.17	-2.84	-2.49	-2.08	-2.48	-2.45	-2.06	-1.62	-2.15
IPO1609_0094	IPO_02163	1.28	1.79	1.71	1.85	1.78	1.87	1.40	1.84	0.87	0.90	0.82	1.13	-4.89	-5.88	-8.63	-3.12	-6.43	-2.21	-6.61	-5.05	-4.96	ND	ND	-4.79	-4.50	-10.42	-7.18	-5.36	-7.00	-4.55	-4.56	-4.80
IPO1609_0095	IPO_02165	1.05	1.41	1.57	1.13	1.00	0.84	0.91	0.82	1.29	0.50	0.90	0.26	-2.99	-2.70	-2.37	-2.41	-2.55	-1.60	-2.57	-1.81	-2.00	-1.44	-2.04	-2.43	-2.03	-1.98	-1.15	-1.68	-1.73	-1.52	-1.76	-1.71
IPO1609_0096	IPO_02166	1.67	1.51	1.42	2.18	2.16	2.30	1.63	1.90	1.18	1.44	1.75	1.63	-4.70	-7.21	ND	-4.37	-6.62	ND	-9.60	-4.73	-3.33	-3.69	ND	-4.78	-9.08	-9.66	-7.87	-7.35	-9.30	-5.30	-4.38	-4.63
IPO1609_0098	IPO_02168/02169	1.11	1.99	1.91	1.82	1.00	0.84	1.45	1.18	1.48	1.46	1.38	1.57	-3.64	-5.06	-9.13	-3.98	-4.63	-1.92	-6.59	-4.42	-2.23	-2.72	-2.86	-1.99	-2.62	-4.09	-3.35	-3.38	-3.38	-2.12	-2.77	-1.93
IPO1609_0099	IPO_02168/02169	1.65	2.95	2.65	2.10	1.76	1.98	1.54	1.81	1.49	1.51	1.77	1.61	-3.75	-3.25	-2.22	-1.09	-4.76	-5.46	-5.28	-3.34	-3.95	-1.48	-1.57	-3.42	-3.98	-4.41	-3.59	-4.36	-2.75	-2.42	-3.48	-3.46
IPO1609_0111	IPO_02448/02449	1.40	1.13	1.29	1.41	1.70	1.78	1.54	1.51	0.60	1.38	1.41	1.53	-4.92	-6.89	-5.98	-4.94	-3.96	-3.71	-4.70	-4.42	-1.89	-2.18	-2.39	-1.92	-1.90	-1.78	-1.56	-2.07	-1.72	-2.02	-2.59	-2.22
IPO1609_0028	IPO_02922/02923	1.44	1.85	1.66	1.72	3.60	3.62	1.68	2.69	1.08	0.87	1.74	1.53	-4.94	-6.12	-8.86	-4.92	-6.25	ND	1.05	-5.21	-5.70	-4.31	-6.52	-5.05	-6.01	-8.53	-4.69	-6.06	-6.35	-5.36	-5.43	-5.18
IPO1609_0027	IPO_02923	1.12	1.71	1.71	1.29	1.61	1.78	1.22	1.52	0.78	1.15	1.35	1.36	-4.73	-7.47	-5.86	-3.98	-5.89	-3.21	-5.16	-4.54	-3.34	-5.76	-6.16	-4.69	-6.35	-8.80	-7.39	-5.50	-6.07	-3.92	-4.70	-3.72
IPO1609_0026	IPO_02924	1.33	1.69	1.53	1.47	2.45	2.81	1.47	2.22	0.82	1.00	1.87	1.35	-5.67	-8.57	ND	-4.51	ND	ND	-2.23	-4.69	-4.58	-5.49	-5.98	-4.58	-4.33	-7.03	-4.66	-4.46	-4.60	-4.00	-3.94	-4.00
IPO1609_0025	IPO_02924/02925	1.15	1.86	1.82	1.19	1.38	1.83	1.33	1.50	0.90	0.90	1.42	1.21	-4.19	-6.56	-6.38	-3.64	-5.23	-3.47	-5.13	-4.02	-2.84	-3.96	-5.32	-3.82	-4.11	-5.95	-3.64	-3.11	-4.06	-2.69	-3.45	-1.81
IPO1609_0023	IPO_02927	1.07	2.30	2.29	1.28	1.00	1.85	1.37	1.61	1.04	0.92	1.51	1.43	-5.22	-6.17	-7.22	-2.91	-6.73	-4.18	-5.18	-4.47	-4.05	-5.65	-6.30	-5.96	-7.21	-8.81	-5.24	-4.77	-6.75	-3.69	-4.23	-3.93
IPO1609_0022	IPO_02927/02928	0.97	2.02	1.97	1.23	1.18	1.64	1.28	1.26	0.97	0.48	1.42	1.32	-4.08	ND	ND	-4.15	-4.17	-6.87	-6.89	-4.34	-2.53	-3.30	-3.99	-2.34	-1.27	-2.40	-2.51	-1.97	-2.05	-1.72	-1.83	-1.84
IPO1609_0021	IPO_02930/02931	1.39	1.57	1.52	1.30	1.22	1.73	1.46	1.32	1.17	1.73	2.17	1.45	-5.74	ND	ND	-4.88	-8.78	-4.67	ND	-5.63	-6.08	-5.60	-8.75	-5.53	-7.65	-10.19	-9.79	-6.22	-9.86	-5.86	-7.34	-6.38
IPO1609_0018	IPO_02934/02935	1.42	1.63	1.54	1.39	2.44	2.78	1.50	1.97	0.97	1.12	1.68	1.36	-4.99	ND	-7.37	-3.95	-4.02	ND	ND	-4.81	-4.67	-4.44	-6.99	-4.60	-7.08	-8.98	-5.31	-5.57	-7.16	-5.83	-5.77	-4.49
IPO1609_0019	IPO_02934/02935	1.46	1.51	1.38	1.44	2.16	2.61	1.53	1.75	0.80	1.38	1.63	1.37	-5.07	ND	-7.49	-4.63	-3.85	-3.78	ND	-5.33	-5.84	-5.55	-6.37	-5.85	-7.68	-10.38	-7.77	-8.04	-9.83	-5.15	-8.68	-5.85
IPO1609_0563	IPO_03105/03107	1.33	1.83	1.68	1.22	0.86	1.36	1.43	1.45	1.63	0.79	1.68	1.57	-5.91	-6.71	-8.86	-3.95	-7.84	-9.04	ND	-5.93	-4.43	-3.30	-3.83	-3.62	-2.76	-6.07	-3.46	-4.45	-4.29	-4.76	-4.55	-4.06
IPO1609_0508	IPO_03123	1.35	1.35	1.11	1.42	1.07	1.29	1.44	1.37	1.35	1.24	1.48	1.37	-8.44	-8.35	-6.65	-6.26	-9.07	ND	-11.20	-8.66	-4.01	-3.61	-0.51	-5.76	-5.73	-6.27	-5.68	-5.99	-6.01	-5.66	-5.97	-5.56
IPO1609_0557	IPO_03132	1.35	1.84	1.59	1.52	1.10	1.12	1.58	1.38	1.09	1.17	1.45	1.15	-3.46	-4.27	-6.00	-2.47	-5.63	-4.66	-6.50	-3.16	-2.64	-3.80	-3.01	-2.89	-7.81	-6.86	-5.09	-2.97	-4.07	-2.23	-2.75	-2.40
IPO1609_0524	IPO_03302	1.15	1.16	1.16	1.15	1.01	-0.19	1.16	1.17	1.43	0.56	1.44	1.19	-5.14	-7.18	-9.25	-4.97	-8.75	-3.96	-6.12	-2.26	-4.20	-4.36	-4.13	-1.43	-1.43	-10.14	-4.42	-6.15	-5.05	-4.55	-4.22	-4.49
IPO1609_0522	IPO_03304/03305	1.51	1.61	1.30	1.21	1.81	0.27	1.48	1.61	1.21	0.79	1.64	1.48	-5.32	-7.75	-6.00	-4.93	-6.49	-5.30	-7.69	-5.14	-5.45	-5.67	-9.69	-4.13	-5.86	-10.02	-6.50	-8.31	-9.81	-7.50	-6.94	-4.76
IPO1609_0523	IPO_03304/03305	1.38	1.29	1.32	1.15	0.99	-0.45	1.34	1.13	1.07	1.64	1.37	1.45	-3.74	-9.95	-3.58	-4.40	-6.00	-6.23	-4.63	-3.24	-2.95	-2.92	-5.05	-3.09	-3.52	-4.66	-4.71	-3.46	-4.46	-3.14	-3.98	-3.18
IPO1609_0521	IPO_03306	1.38	1.47	1.25	1.39	1.87	2.26	1.62	1.94	0.68	1.22	2.00	1.47	-5.94	-7.05	-7.06	-5.59	-7.78	-9.55	-9.72	-6.22	-6.22	0.48	-7.49	-5.18	-9.27	-9.47	-7.92	-5.64	-9.27	-6.26	-5.31	-5.39
IPO1609_0520	IPO_03306/03307	1.14	1.20	1.24	1.43	1.78	1.62	1.42	1.68	1.13	1.17	1.39	1.36	-2.92	-7.52	-6.04	-4.34	-6.86	-5.51	-6.84	-2.15	-4.12	0.95	-6.64	-2.75	-4.51	-8.88	-3.99	-4.23	-7.64	-4.58	-4.82	-3.70
IPO1609_0457	IPO_03656	1.54	1.62	1.61	1.36	-2.55	2.35	1.38	1.82	0.99	1.17	1.69	1.45	-4.63	-7.77	-8.87	-4.40	ND	ND	ND	-4.83	-4.33	ND	ND	-4.72	-9.15	-9.11	-8.68	-6.06	-8.32	-4.76	-6.58	-4.48
IPO1609_0456	IPO_03659	1.19	1.76	1.74	1.40	-2.22	1.17	1.42	1.48	1.55	1.49	1.56	1.52	-4.22	-5.00	-4.75	-2.93	-4.90	ND	-5.31	-4.45	-4.47	-4.96	-4.27	-4.21	-6.57	-5.73	-4.73	-3.91	-4.15	-2.66	-3.94	-4.00
IPO1609_0455	IPO_03660/03661	1.47	1.52	1.40	1.51	-3.23	2.40	1.56	2.03	1.11	1.70	1.92	1.54	-5.85	-7.75	ND	-5.40	-5.64	ND	ND	-5.88	-4.82	-3.38	ND	-6.24	-9.36	-10.79	-7.96	-6.36	-8.16	-5.24	-5.71	-5.15
IPO1609_0435	IPO_03741/03742	1.56	1.08	1.04	1.60	-1.53	1.63	1.73	1.73	1.13	1.45	1.59	1.61	-4.62	ND	ND	-3.61	-6.20	ND	ND	-5.06	-4.24	-7.28	ND	-3.03	-5.53	-9.87	-9.90	-4.20	-9.64	-4.37	-4.85	-4.47
IPO1609_0434	IPO_03742	1.49	1.08	1.16	1.29	-1.40	0.95	1.46	1.17	1.21	1.09	1.48	1.47	-4.73	-6.76	-5.87	-3.80	-6.61	-4.54	-6.47	-5.00	-4.28	-4.67	-6.93	-1.64	-1.49	-10.03	-6.96	-7.02	-9.22	-5.74	-6.99	-5.31
IPO1609_0420	IPO_03816	1.22	1.68	1.53	1.14	0.58	0.72	1.25	1.04	1.81	0.74	1.57	1.37	-5.20	-5.17	-6.71	-2.25	-6.02	ND	-5.84	-4.45	-3.51	-5.99	1.39	-3.61	-2.71	-3.67	-2.51	-4.72	-3.34	-3.84	-3.29	-3.74
IPO1609_0418	IPO_03816/03817	1.35	1.62	1.72	1.61	1.24	1.30	1.53	1.50	1.47	1.73	1.56	1.55	-4.18	-5.85	-6.82	-1.97	-6.05	ND	-4.79	-4.56	-2.65	-4.07	2.19	-2.61	-2.97	-7.17	-6.60	-4.98	-8.62	-4.44	-4.33	-4.12
IPO1609_0419	IPO_03816/03817	1.19	1.61	1.57	1.42	1.93	2.00	1.58	1.72	0.99	1.01	1.71	1.47	-5.81	-5.90	-8.73	-5.11	-6.75	ND	-8.88	-5.27	-3.74	-4.17	-4.44	-1.38	-2.13	-3.62	-3.45	-3.03	-3.75	-2.95	-2.97	-3.02
IPO1609_0383	IPO_03999/04000	1.49	2.17	1.57	1.61	1.41	1.55	1.68	1.74	1.19	0.90	1.71	1.75	-4.21	-6.12	ND	-3.86	ND	ND	ND	-4.31	-5.46	1.77	ND	-4.63	-7.78	-9.17	-8.51	-5.00	-8.42	-6.12	-3.98	-4.69
IPO1609_0382	IPO_04000	1.68	6.36	1.47	1.59	1.64	1.94	1.52	1.85	1.85	ND	1.78	1.66	-4.23	-7.35	-7.11	-4.48	ND	-4.75	ND	-4.58	-4.41	1.79	-5.01	-4.38	-3.64	-8.97	-5.99	-9.05	-6.86	-5.15	-5.33	-4.92
IPO1609_0376	IPO_04000/04001	1.28	5.97	1.56	1.28	1.51	1.35	1.34	1.31	1.47	1.41	1.34	1.47	-2.99	-5.34	-4.37	-4.19	-5.02	-3.81	-4.26	-2.06	-3.08	1.49	-3.60	-2.86	-2.65	-3.43	-2.90	-3.31	-2.34	-2.68	-2.94	-2.63
IPO1609_0377	IPO_04000/04001	1.44	6.22	1.40	1.56	2.27	2.31	1.62	2.09	1.22	1.46	1.80	1.57	-6.23	-7.11	ND	-5.16	-8.42	ND	ND	-5.73	-4.02	1.37	ND	-5.28	-8.28	-8.10	-6.05	-5.27	-5.41	-5.78	-4.81	-4.95
IPO1609_0378	IPO_04000/04001	1.43	6.31	1.53	1.46	2.03	2.21	1.72	2.10	1.49	1.26	2.01	1.64	-5.16	-4.05	-4.01	-5.03	-9.12	ND	-7.38	-5.52	-4.08	0.68	-5.22	-4.59	-6.69	-5.19	-3.65	-4.73	-4.30	-4.48	-4.57	-4.00
IPO1609_0379	IPO_04000/04001	1.45	6.45	1.64	1.30	2.17	2.33	1.53	1.73	1.14	1.31	1.68	1.52	-4.68	-5.85	ND	-4.10	ND	ND	ND	-4.74	-4.35	0.65	ND	-5.21	-8.44	-9.50	-6.65	-4.66	-8.11	-8.00	-7.07	-4.39
IPO1609_0380	IPO_04000/04001	1.20	6.64	1.70	1.23	1.12	1.45	1.38	1.54	1.60	0.20	1.74	1.37	-5.63	-7.13	ND	-5.07	ND	-3.90	-7.85	-5.80	-5.14	1.25	-6.16	-5.75	-6.54	-9.80	-5.90	-7.94	-4.92	-8.32	-7.79	-6.36
IPO1609_0381	IPO_04000/04001	1.36	6.35	1.69	1.69	2.33	2.22	1.90	2.03	1.21	1.14	1.52	1.84	-5.28	-7.92	ND	-2.61	ND	ND	ND	-5.18	-5.26	1.60	ND	-5.03	-6.22	-9.26	-6.63	-6.43	-7.09	-5.84	-5.21	-4.25
IPO1609_0375	IPO_04001	1.13	6.34	1.55	1.41	1.28	1.36	1.50	1.66	1.06	0.87	2.75	1.47	-4.57	-5.86	ND	-2.77	-6.04	ND	ND	-4.91	-5.40	1.63	ND	-4.05	-8.38	-8.26	-7.35	-5.80	-7.54	-4.26	-4.48	-3.99
IPO1609_0374	IPO_04002	1.18	5.64	1.06	0.96	1.70	1.20	1.23	1.21	1.10	0.95	1.20	0.64	-4.61	-3.39	-2.51	-2.34	-2.84	-5.56	-2.96	-2.59	-2.88	0.93	-2.65	-3.03	-2.34	-2.37	-1.70	-2.69	-2.07	-1.93	-1.94	-2.48
IPO1609_0373	IPO_04003	1.46	6.22	1.53	1.80	1.31	1.21	1.80	1.47	1.57	1.24	2.19	1.62	-3.52	-5.02	-5.59	-2.16	-6.48	ND	-6.27	-4.30	-3.90	1.82	ND	-1.84	-2.91	-3.22	-1.97	-2.30	-2.19	-1.43	-2.14	-1.16
IPO1609_0372	IPO_04003/04004	1.30	6.54	1.96	1.72	1.35	0.95	1.74	1.59	1.98	1.08	1.64	1.85	-4.18	-4.45	-5.89	-1.81	-5.66	ND	-5.02	-3.90	-3.36	1.95	-4.59	-4.05	-4.33	-6.51	-3.12	-5.20	-3.51	-3.52	-5.16	-4.04
IPO1609_0371	IPO_04004	1.54	1.22	1.16	1.46	1.46	1.57	1.60	1.64	1.23	1.65	1.56	1.35	-3.53	-3.34	-8.43	-4.43	-6.53	-7.06	-4.69	-4.66	-4.35	-5.87	-5.45	-0.63	-3.26	-6.89	-4.16	-3.56	-4.44	-3.45	-3.20	-3.32
PT04834A	IPO_04353	1.22	2.04	1.89	1.23	1.22	1.27	1.49	1.39	1.03	0.51	1.34	1.52	-3.61	-3.95	-4.36	-1.55	-3.14	-4.85	-3.15	-4.14	-3.27	-4.47	-2.41	-3.51	-4.37	-6.01	-3.66	-3.97	-4.67	-3.38	-3.21	-2.94
IPO1609_0264	IPO_04521	1.62	1.47	1.53	2.06	3.33	3.74	2.11	2.63	0.85	0.87	1.93	1.68	-4.90	-8.43	-6.29	-5.10	ND	-8.85	-7.68	-4.45	-5.63	-5.09	-7.06	-5.16	-4.97	-6.29	-4.46	-5.17	-4.86	-5.64	-5.87	-4.30
IPO1609_0263	IPO_04523	1.36	1.75	1.95	1.49	1.60	1.78	1.52	1.80	0.83	0.99	1.77	1.37	-5.47	ND	ND	-4.34	ND	ND	ND	-5.96	-4.32	-4.24	-3.92	-4.39	-4.57	-5.67	-3.18	-3.69	-3.95	-4.24	-3.80	-2.84
IPO1609_0262	IPO_04524	1.78	1.57	1.72	1.78	1.96	2.01	1.81	1.74	1.21	1.24	1.74	1.70	-4.40	-7.41	-7.32	-4.65	-5.71	ND	ND	-4.22	-4.19	-5.09	-7.13	-4.28	-5.52	-7.16	-4.33	-5.09	-4.67	-4.98	-4.45	-4.31
IPO1609_0261	IPO_04525	1.63	1.96	1.98	1.50	1.75	2.10	1.72	1.87	1.00	0.82	1.79	1.56	-5.38	ND	-8.72	-3.53	ND	-4.31	-9.97	-5.54	-5.10	-5.92	ND	-5.31	-7.78	-10.05	-5.26	-5.49	-6.56	-5.78	-4.90	-4.63
IPO1609_0260	IPO_04526	1.30	1.88	1.81	1.19	0.97	1.38	1.40	1.25	0.81	0.49	1.56	1.34	-4.81	ND	ND	-5.25	-3.64	-6.55	ND	-5.48	-4.39	-3.94	-7.85	-5.75	-6.56	-6.92	-5.58	-6.29	-4.67	-6.52	-5.27	-5.20
IPO1609_0254	IPO_04530	1.63	1.25	1.37	2.14	2.46	3.05	1.82	2.47	0.91	0.54	1.89	1.67	-4.85	-1.91	-4.77	-5.44	-5.37	-6.45	-9.90	-5.09	-4.85	-4.72	-2.38	-4.94	-5.50	-7.49	-6.32	-6.54	-6.59	-8.06	-6.53	-4.95

Validation of candidate IIB-1 specific genomic regions

The list of candidate IIB-1 specific genomic regions detected by CGH was established based on the analysis of a limited number of strains. Therefore, we validated this list on a larger collection of strains representative of the diversity found in the *R. solanacearum* species complex, including 9 IIB-1 strains (7 of which were also used for CGH experiments) and 32 non IIB-1 strains (7 of which were also used for CGH experiments) (Table 4-1). This validation was conducted by PCR amplification of one genomic fragment from each candidate IIB-1 specific gene. All tested DNA could be amplified using the multiplex PCR for the *R. solanacearum* phylotype identification as described by Fegan and Prior (2005), thereby confirming that the tested strains actually correspond to *R. solanacearum* isolates (Figure 4-1).

Each primer pair that gave a positive amplification from non IIB-1 strains or a negative amplification from IIB-1 strains was excluded from the final list of IIB1sgr (Table 4-3). The results validated the specificity of 85 IIB1sgr, including 70 genes and 15 intergenic sequences. These regions are organized in 9 gene clusters and 6 individual genes. Among these, 29 genes were predicted to be parts of mobile genetic elements (bacteriophage or insertion sequences) and 34 were classified as ACURs (Alternate Codon Usage Regions) according to Salanoubat et al. (2002).

Use of IIB1sgr as targets for specific detection of IIB-1 strains

A subset of 5 genes belonging to clusters 3 and 6 were used as targets for the development of new PCR methods for specific detection of IIB-1 *R. solanacearum* strains (Table 4-4). These genes were selected from the final list of IIB1sgr since they were not predicted to be part of mobile genetic elements or ACURs, thereby sustaining their reliability as specific markers for detection. For these genes, additional primers were designed to amplify PCR products ranging from 300 to 600-bp, and optimal PCR conditions determined.

All PCR assays tested were able to amplify a single product of the expected size, but primer combinations differed in specificity and amplification yield. Based on these results, we selected the following primer combination for further evaluation: 02103F1-02103R2 (Figure 4-2A). In the present work we used this primer set in combination

with universal primers for *R. solanacearum* 759-760 (Opina et al., 1997) for the development of a multiplex PCR assay for detection of all *R. solanacearum* strains and simultaneous specific identification of IIB-1 potato brown rot strains (Figure4-2B). This multiplex PCR assay was designated as Mx-RsIIB1.

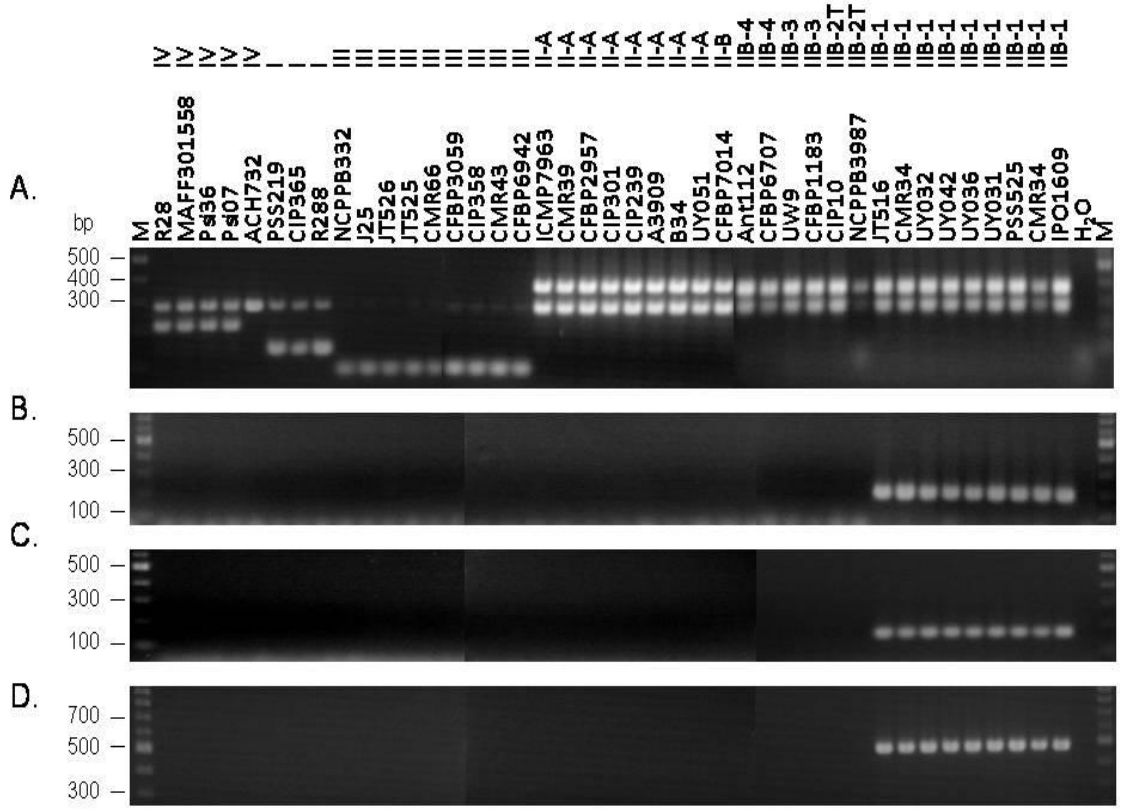


Figure 4-1. Representative results for the validation of specific genes for IIB-1 *Ralstonia solanacearum* strains. This validation was conducted by PCR amplification of one genomic fragment from each gene using the PCR primers given in Table 4-3 and genomic DNA from 9 IIB-1-strains, 32 non IIB-1 strains, and H_2O as a negative control. The ID and phylotype of the strains are given for each lane. A. Multiplex PCR as described by Fegan and Prior (2005) for the *R. solanacearum* phylotype identification. B. PCR amplification of a 206-bp fragment of the IPO_00043 gene. C. PCR amplification of a 171-bp fragment of the IPO_02090 gene. D. PCR amplification of a 491-bp fragment of the IPO_02155 gene. These three last fragments could be amplified from IIB-1 genomic DNA only. M: molecular weight marker.

Table4- 3. Lists of *Ralstonia solanacearum* IIB-1 specific regions identified through microarray comparative genomic and validated through PCR amplification using specific forward (L) and reverse (R) primers design for each gene. Black and grey boxes indicate mobile genetic elements and alternative codon usage regions (ACURs) respectively.

IIB1 specific gene clusters					
Cluster	**Gene ID**	**Function**	**L_PCR primer**	**R_PCR primer**	**PCR product size (bp)**
1	IPO_00043	Hypothetical protein	CTGAATTCGAAAAGGATAGAGCA	CTCGACAAACTCTTGCAACTGAC	206
1	IPO_00044	Hypothetical protein	**CTATGCAGAAGCGTTGCTTGTT**[b]	CTTTAGCGAGCACAAGATTGAGT	191
1	IPO_00045	ParB-like nuclease	**GAGATCGTTGGAAACATCAAGAC**[b]	GTGAACCACTATTGCCGGTATC	165
1	IPO_00046[a]	Recombinase			
1	IPO_00046/00047	intergene			
2	IPO_00232/00233	intergene			
2	IPO_00233	Hypothetical protein	AGAACTGCCAAGTTCGACTACCT	**CATTCCAACGTTCAGATGGTTAT**[b]	207
2	IPO_00234	Thymidylate synthase	GCAGAAAAGATATCCCCTGCAC	**TTCGAGTACAAATGTAGGCTTCC**[b]	214
3	IPO_00874/00875	intergene			
3	IPO_00875	Hypothetical protein	GATCAGATGGAGCAAAGAACACT	TATTGAAACTCTTCACGGGTCAT	221
3	IPO_00875/00876	intergene			
3	IPO_00876	Hypothetical protein	TTTCGACCAAGAAAAGCATAGAG	ATTTCTGTGCCCACTACGAACTA	238
3	IPO_00877	Hypothetical protein	TGGTGTTCTAACTGTGGAAGGTT	TACCGCCAGTCATATCAGTTCTT	163
3	IPO_00878	Hypothetical protein	**ATTAGACTGATCAAGGCATGGAA**[b]	CCTTCATTATTGAGACGGTCAAG	152
3	IPO_00878/00879	intergene			
3	IPO_00879	Hypothetical protein	**ATGTTTGTGCTACTGGTCAGTCC**[b]	CCTTCACTTGCAGATAATGGAAC	223
3	IPO_00880[a]	Hypothetical protein			
3	IPO_00881	MobA-related protein	AAAGAAGCTCAAGGAGATCAAGG	**AACAGCAGGTTGTGATACTGCAT**[b]	201
3	IPO_00882[a]	transposase			
3	IPO_00883[a]	Transposase (trucated proitein)			
3	IPO_00883/00884	intergene			
4	IPO_01258/01259	intergene			
4	IPO_01259	Hypothetical protein	**TGAAATGCTCAAAGACAAACAGA**[b]	ATCGTACAGGTCATTGCCAAAT	235
4	IPO_01260	transposase	TACAACCTGAAGAGGATCTCGAA	**AAAGCCGGTCATAGAGGACATAG**[b]	225
5	IPO_01311	Hypothetical protein	**CAACCAGACCATCTACAAGATCC**[b]	GCTTCATACTCAAATCGAACACC	165
5	IPO_01312	Hypothetical protein	AACTCCAACTTGCTTGACTGTTC	GGATGAACTTCGTTCGATTGAG	208
5	IPO_01313[a]	recombinase			
5	IPO_01314	Tyrosine recombinase	GAAGCTCGGTGATATCGAAAC	GGTGATCGCTGTCGATAATTT	287
6	IPO_02100/02101	intergene			
6	IPO_02101[a]	Hypothetical protein			
6	IPO_02102	Hypothetical protein	GTATCAGAAAGGCCAGCTACACA	**CTGATTGCCAATATTCGATTCTC**[b]	207
6	IPO_02103[a]	N-6 Adenine-specific DNA methylase			
6	IPO_02103/02104	intergene			

Table 4-3. Continued

IIB1 specific gene clusters					
Cluster	**Gene ID**	**Function**	**L_PCR primer**	**R_PCR primer**	**PCR product size (bp)**
7	IPO_02140	Hypothetical protein	AAGGGCAATGGCTTTTTCTGT	**GAGACTGATAAATCAGCGTTTCC**[b]	153
7	IPO_02141	Tail fiber protein	GCTTTCTACGTCGCCTCAGTAT	**GTAACGGTCTGATTCTTGAGGTTT**[b]	225
7	IPO_02141/02142	intergene			
7	IPO_02142	Baseplate protein	CCACTACCCCTGTTTCCATTC	AAACCTGTAGTCGTCGTCCTTG	245
7	IPO_02143	Phage baseplate assembly protein	**AGACGTTGGACAACATCAACC**[b]	AGTTCTGTTCGTCGTCGTGAAT	189
7	IPO_02144	Bacteriophage tail protein	**CCAGTATTCCAAGACGCTATCC**[b]	AACGGATACAGCAGCAGGTTT	189
7	IPO_02145	DNA circulation	ATACCCAGCGCGTATTCCAA	**GTTGAGGCAACACCAGACAG**[b]	164
7	IPO_02146	Phage tail tape measure protein TP901, core region	CTTGAGAAAGCTTTTGGTGAAGA	**CTTTGAGACCTTCCCAGGCTAA**[b]	166
7	IPO_02147	Hypothetical protein	TAAGAGCAGGCTATGGACAACAT	AATACCAGCACACAGAAGGTCAG	209
7	IPO_02148	Bacteriophage tail sheath	**CTGATTTCCATGTACCTGCATC**[b]	ACATCAGCACGTTCTTGTAGAGC	235
7	IPO_02149	Hypothetical protein	GACGATGAGTTGCTGAAGTACC	TGAAGGTAATGGTCACAGCTTTT	237
7	IPO_02150	Hypothetical protein	**ATCCGGTTCCTTCTGATGATCT**[b]	CTTCAACTTCACGTGTTCAATCA	124
7	IPO_02151	Hypothetical protein	GCTAGTCCACACGACAAAATCAT	**GTGACTAGCTTGGCGATCTTCT**[b]	163
7	IPO_02152	Mu phage/prophage-related protein	**AGGTGAAGTGCTCGAAATCCT**[b]	CTTCTTCCTTCTCGATGCCTTC	284
7	IPO_02153	TPR-domain protein	TTATGTGGCTTTCTCTGGCAATA	**ACAAACGTCCAGTCGTCAATC**[b]	228
7	IPO_02154	Transmembrane	CATTTATCTCTGGTCTTGGCTTG	**ACAGCCAAACTGACAAGATCG**[b]	173
7	IPO_02154/02155	intergene			
7	IPO_02155	Hypothetical protein	GCATGAAAATGTCTACGTTCCTC	ATGGTGATAGTGCGGAATGAC	491
7	IPO_02156	Transmembrane	**CAGACATGTTCTGCGAAGGAT**[b]	GAGGAACGTAGACATTTTCATGC	283
7	IPO_02157	DNA-binding protein HU-beta (NS1) (HU-1)	TGTAATGACCAAGCAAGAACTCA	**CACGGTGTCAAGAATGGTTTC**[b]	112
7	IPO_02158	Hypothetical protein	ATTTCAAACGCCAAGCCTTTAAC	**GTTCATCGAAAGCGATGTTCTC**[b]	165
7	IPO_02159	Hypothetical protein	GCCTATTTGGACCGAAGAAGAC	ACTTCTTGAGGCATCTCGGTTT	394
7	IPO_02159/02160	intergene			
7	IPO_02160	Lambda repressor-like, DNA-binding	GCAGCATTGATGACAAGTTCC	AGTATTGGTAAAGGCGTTGCAC	339
7	IPO_02161[a]	Hypothetical protein			
7	IPO_02162	MuA-transposase/repressor protein CI-related protein	AAGGCTAAGGGGGAGTAAGTCAT	**AGGTAGTTGCGTACTTGGTCGTA**[b]	548
7	IPO_02163	Hypothetical protein	**AGCAACCGAAGATATCGACCT**[b]	GCCCAAGCGAAATCAACTCTT	284
7	IPO_02164[a]	phage-related DNA-binding protein			
7	IPO_02165	Helix-turn-helix motif	CTTTCCCAGTCAATACATTCCAG	CGTTATCACATAAGCCACATCAA	220
7	IPO_02166	Lipocalin	**AAACAACACGCTGTTGAGCAT**[b]	CTTCTTTGCGCACCAATAATC	195
7	IPO_02167[a]	phage-related protein			
7	IPO_02168[a]	Lytic transglycosylase, catalytic			
7	IPO_02168/02169	intergene			
8	IPO_03302	Hypothetical protein	**CGTGTATATCGGCAGTCAAGAAG**[b]	CTAAGAAATGAAAGGTGGGGTTC	227
8	IPO_03303[a]	Hypothetical protein			
8	IPO_03304[a]	Transmembrane protein			
8	IPO_03304/03305	intergene			
8	IPO_03305[a]	Hypothetical protein			
8	IPO_03306	Hypothetical protein	GGATGACTTGTGTTAGCGACTCT	**GAATACGATCCTCCACAATCAAA**[b]	171
8	IPO_03306/03307	intergene			

Table 4-3. Continued

IIB1 specific gene clusters					
Cluster	**Gene ID**	**Function**	**L_PCR primer**	**R_PCR primer**	**PCR product size (bp)**
9	IPO_04521	Hypothetical protein	TGAAGCACTGTCTATCAACCAGA	TTTGTCCTAGTCACAGCACTGAA	222
9	IPO_04522[a]	Hypothetical protein			
9	IPO_04523	Hypothetical protein	GACTTCGGCTATCTGGAGAAAAT	TCTTAGCAGGTTTAGGCTGAGTG	217
9	IPO_04524	Hypothetical protein	CATCTTCAAGGATGACTCTCTGG	**GAAGAAGTGACCAGGCTGAATTT**[b]	235
9	IPO_04525	Helicase-like protein	ACTCAGTGACGAAGAGGTTGAAG	ACGAGTAGCTTCAATGGTGTCTT	250
9	IPO_04526	Hypothetical protein	ACACTATGCCTGCTGACTTGAA	AGGTGACTTCAACAATGTTAGGC	169
IIB1 specific individual genes					
10	IPO_01058	Hypothetical protein	**CGAGCTCATCGTTATCGACAT**[b]	AAGCTCTTGGACTAGGACGATCT	140
11	IPO_01362	Hypothetical protein	**AATTGGGTATACGTGATCTGTGG**[b]	TCGGGTAAGACGAAGCTGACTA	280
12	IPO_02090	Hypothetical protein	CAATAGAAATTGCCGAGGTGATA	**CCTTGATAAGGATGTTCAACGAC**[b]	171
13	IPO_03123	Transposase	GAACGGAGCCATAGTGATGAAG	AGAGTCGCTAACACAAGTCATCC	946
14	IPO_03132	Helicase,6 related protein	AGGGAATCAAATCGCTCATCT	AGAAGAAGCCCATGATGACAGAG	226
15	IPO_04004	Hypothetical protein	CTTGACGTCTGACAACCAAGTAG	ATAAGATAAACAGGTCGGCCTTC	342

[a] Added genes correspond to the genes that were included into the list based on their location within clusters of IIB-1 specific genes.
[b] Primer sequences in bold correspond to part of the oligonucleotides spotted on the microarray.

Table 4-4. Primers designed for IIB-1 specific genes selected as target for PCR detection of potato brown rot *Ralstonia solanacearum* strains

Gene ID	Forward primer	Reverse primer	Primer combination	Product size (bp)
RSIPO_00876	00876F1: GGATTCAAGGTATCGCCAGA	00876R1 : CATAGCCGCTTCTTCTTTGG	00876F1 – 00876R1	342
		00876R2 : CAAGCCTGAAGATTCCGAAG	00876F1 – 00876R2	377
RSIPO_00877	00877F1: ACCTGATACCCAATGCTTCG	00877R1 : TCCATTCCCCATCGTCATAG	00877F1 – 00877R1	391
RSIPO_00878	00878F1: TAGAGCCACTGCTGCTGAGA	00878R1: GCATATTTCGCCACCTTCAT	00878F1 – 00878R1	448
	00878F2: GCTTGTCTGCCGACTACCTC	00878R2: TACCTCCGTGCTTACCATCC	00878F1 – 00878R2	545
			00878F2 – 00878R1	294
			00878F2 – 00878R2	391
RSIPO_00879	00879F1 : AGATGGTGGAATTGGTGGAG	00879R1 : ACCGCAAATGGAGAAACAAC	00879F1 – 00879R1	260
	00879F2 : ATGACCCAGTAGGCAAGACC	00879R2 : GTCCTACGGCTTCAACTTCG	00878F2 – 00879R1	481
			00878F2 – 00879R2	329
RSIPO_02103	02103F1 : ATTGCCCACTACTTGGAACG	02103R1: TGGAATGCAAACTCAAGCTG	02103F1 – 02103R1	433
	02103F2 : CAAGAACAACTACCGCAGCA	02103R2: AACTACGAGGGTGGTTGCAG	02103F1 – 02103R2	329
			02103F2 – 02103R1	347
			02103F2 – 02103R2	243

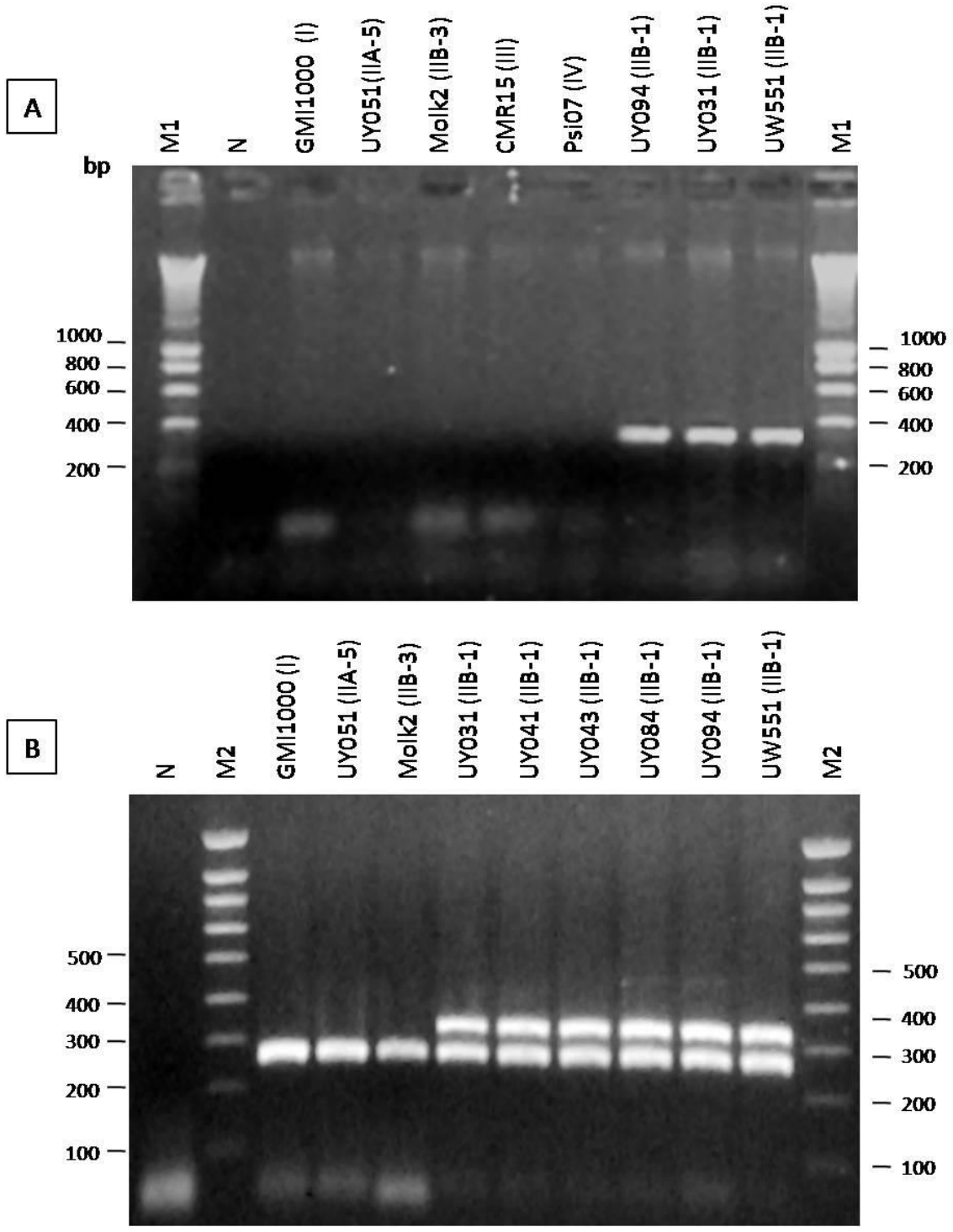


Figure 4-2. Evaluation of new PCR based methods for specific detection of IIB-1 *Ralstonia solanacearum* strains. A: PCR amplification of a 329-bp fragment using IIB-1 specific primers 02103F1-02103R2. B: Multiplex PCR assay (Mx-RsIIB1) using IIB-1 specific primers 02103F1-02103R2 in combination with universal *R. solanacearum* primers 759-760 (Opina et al., 1997) which amplify a 282-bp product for all *R. solanacearum* strains. PCR reactions were conducted with genomic DNA from different *R. solanacearum* strains, and water as negative control (N). The ID and phylotype of strains are given for each lane. M1: molecular weight marker (Smart Ladder Eurogentec, 10-Kb). M2: molecular weight marker (Smart Ladder Eurogentec, 1-Kb).

Specificity and sensitivity of the Mx-RsIIB1 assay

The specificity of primers 02103F1-02103R2 was first evaluated in silico, with Primer-BLAST tool, revealing no significant matches with non IIB-1 *R. solanacearum* strains, as well as with other *Ralstonia* species or related genera. To experimentally test the specificity of these primers, multiplex PCR assay was carried out with genomic DNA of 10 IIB-1 *R. solanacearum* strains, 45 strains representing others phylotypes and sequevars of *R. solanacearum* species complex, and 10 strains corresponding to other genera obtained from environmental samples (Table 4-1). As expected, all 55 *R. solanacearum* strains tested yield an amplification product of 282-bp with primers 759-760. In addition, a specific DNA fragment of 329-bp was obtained for all IIB-1 *R. solanacearum* strains (Figure 4-3). No amplification product was obtained from DNA of other bacteria tested.

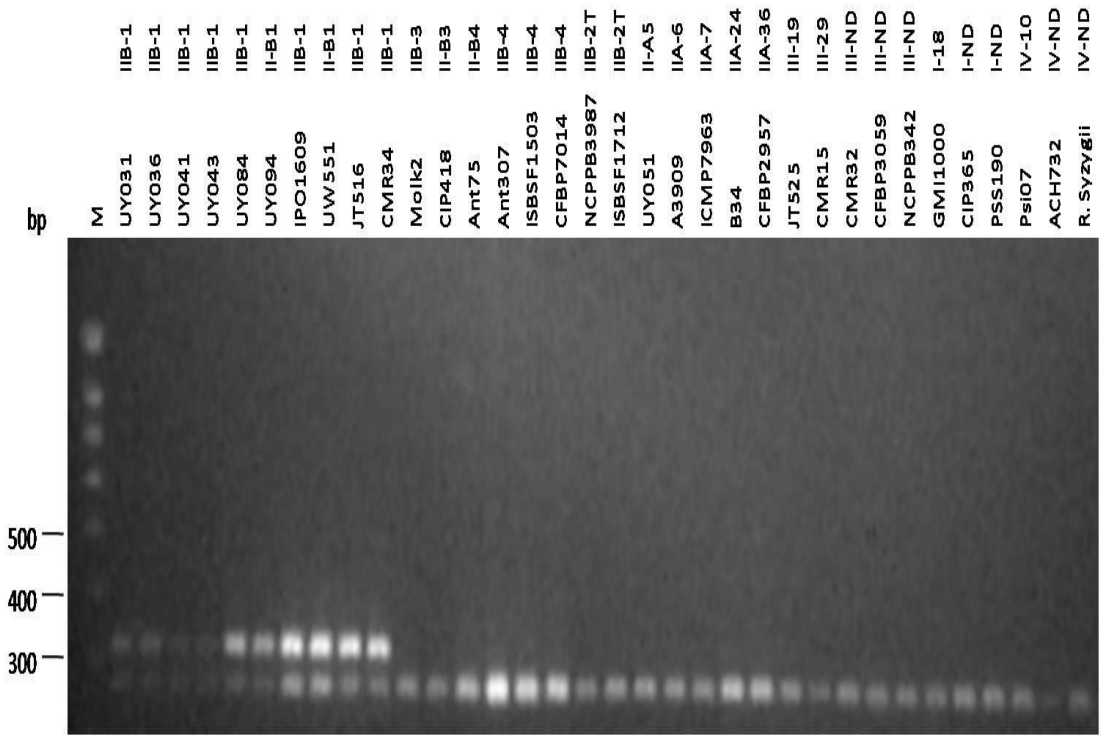


Figure 4-3. Specificity evaluation of a multiplex PCR assay for detection of *Ralstonia solanacearum* and simultaneous identification of IIB-1 strains. The ID and phylotype of strains are given for each lane. M: molecular weight marker (Smart Ladder Eurogentec, 1-Kb).

Sensitivity of amplification using IIB-1 specific primers was first evaluated with serially diluted genomic DNA samples from strain UY031 (IIB-1). Positive amplification was obtained up to 50 pg of DNA per PCR reaction (Figure 4-4). The detection limit was the same for the multiplex assay Mx-RsIIB1. The sensitivity threshold of Mx-RsIIB1 assay was also examined by amplification of pure *R. solanacearum* suspensions in saline solution and the test was positive up to the 10^{-4} dilution. Accordingly, detection limit was $9x10^4$cfu/ml, equal to about 450 cells per PCR reaction (Figure 4-5).

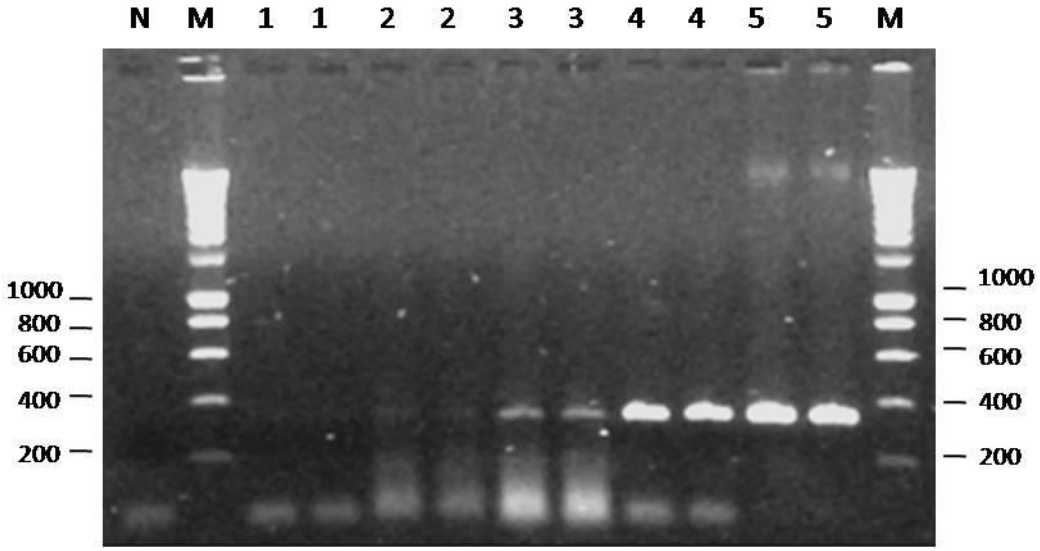


Figure 4-4. Sensitivity evaluation of a PCR assay using *Ralstonia solanacearum* IIB-1 specific primers 02103F1-02103R2 through amplification of serial dilutions of genomic DNA of strain UY031 (IIB-1). DNA samples were assayed in duplicate in the following amount per PCR tube, 1: 5 pg, 2: 50 pg, 3: 0.5 ng, 4: 5 ng, and 5: 50 ng. N: negative control without DNA. M: molecular weight marker (Smart Ladder Eurogentec, 1-Kb)

Application of Mx-RsIIB1 assay for detection of *R. solanacearum* in potato tubers

To assess the sensitivity of detection of Mx-RsIIB1 in infected potato tubers, extracts were prepared and inoculated with 10-fold serial dilutions of *R. solanacearum* (IIB-1). The lowest population level detected by direct multiplex PCR assay was $9x10^6$cfu/ml, which correspond to $4,5x10^4$ cfu per PCR reaction (Figure 4-6). The sensitivity of Mx-RsIIB1 was increased significantly by performing an enrichment step in mSMSA broth prior to amplification. Following this BIO-Mx-RsIIB1 procedure the sensitivity threshold increased to $1,2x10^3$cfu/ml of potato tuber extract (Figure 4-7).

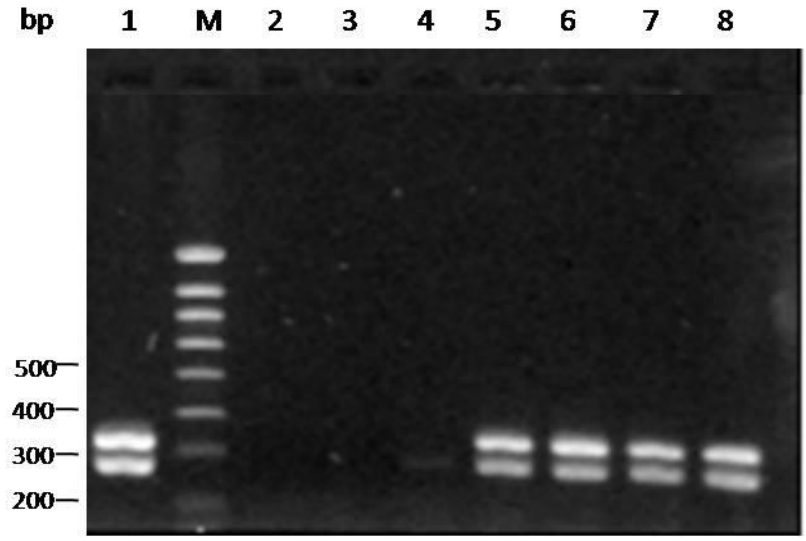


Figure 4-5. Sensitivity of multiplex Mx-RsIIB1 assay in pure suspensions of IIB-1 *Ralstonia solanacearum* cells. A cell suspension of strain UY031 (IIB-1) was adjusted to 0.1 OD_{600} in sterile saline solution and 10-fold diluted to 10^{-6}. Suspensions were boiled for 10 min and 5 µl were used for multiplex PCR. Lane 1: genomic DNA of strain UY031 (IIB-1); lanes 2-8: 10-fold serial dilutions of *R. solanacearum* cells (strain UY031) in sterile saline solution ranging from 90 cfu/ml to $9x10^7$ cfu/ml. M: molecular weight marker (Smart Ladder Eurogentec, 1-Kb).

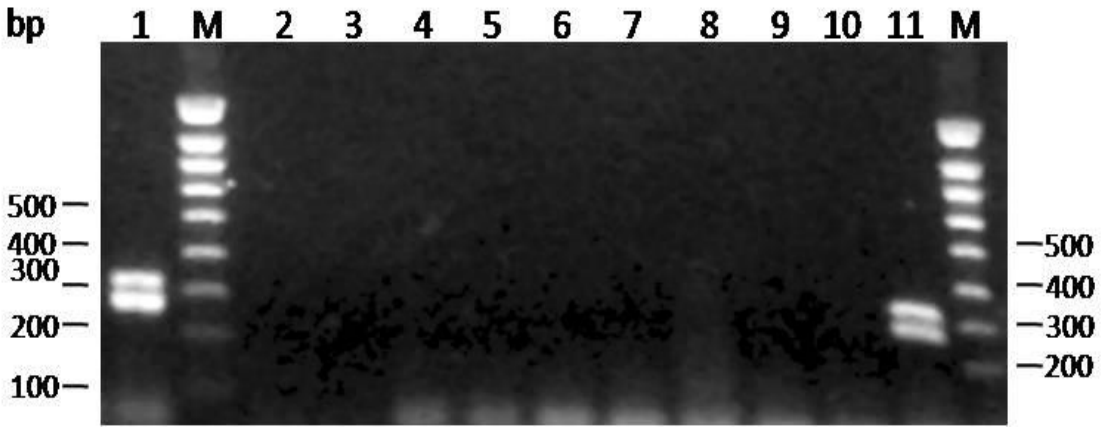


Figure 4-6. Sensitivity of multiplex Mx-RsIIB1 assay for detection of *Ralstonia solanacearum* strains in spiked potato tuber extracts. Healthy potato tuber extracts were mixed with 10-fold dilutions of *R. solanacearum* cells (strain UY031), boiled for 10 min and 5 µl were used directly for multiplex PCR. Lane 1: genomic DNA of strain UY031; lane 2: negative control of PCR (without DNA); lane 3: healthy potato sample (non spiked); lane 4-11: potato extracts inoculated with 10-fold serial dilutions of *R. solanacearum* cells ranging from 1,2 cfu/ml to $1,2x10^7$cfu/ml of potato extract. M: molecular weight marker (Smart Ladder Eurogentec, 1-Kb).

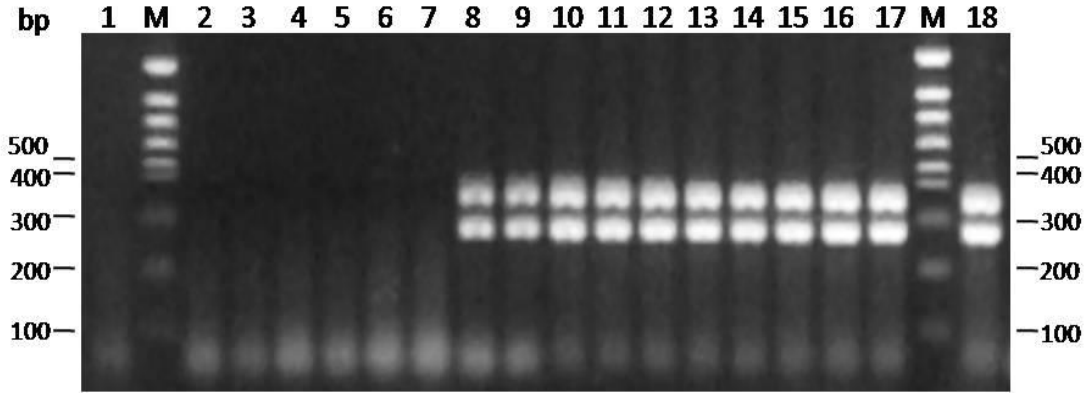


Figure 4-7. Sensitivity of BIO-Mx-RsIIB1 assay for detection of *Ralstonia solanacearum* strains in spiked potato tuber extracts. Healthy potato tuber extracts were mixed with 10-fold dilutions of *R. solanacearum* cells (strain UY031) and 100 µl of each inoculated potato extract were added to 900 µl mSMSA broth and incubated for 48 h at 28ºC. 100 µl of each enriched sample were boiled for 10 min and aliquots (5 µl) were assayed by multiplex PCR. Lane 1: healthy potato sample (non spiked); lane 2-17: duplicated potato extracts inoculated with 10-fold serial dilutions of *R. solanacearum* cells ranging from 1,2 cfu/ml to $1,2x10^7$ cfu/ml of potato extract; 18: genomic DNA of strain UY031 M: molecular weight marker (Smart Ladder Eurogentec, 1-Kb).

DISCUSSION

In the present work, we identified a set of 70 genes and 15 intergenes that were specific to potato brown rot strains from *R. solanacearum* phylotype IIB-1, historically known as race 3, biovar 2 strains. This list was identified by microarray based CGH analysis and subsequent PCR validation with a collection of strains covering the known genetic diversity in *R. solanacearum*. Within the IPO1609 genome, these brown rot strain specific genes are organized in 9 clusters encompassing 2 to 29 genes, and in 6 single genes dispersed in the genome. Among the 70 specific genes, 29 (41%) are parts of mobile genetic elements such as transposases, recombinases or phage proteins and 34 (49%) were classified as ACURs. This suggested that most of these specific genes originate from acquisition of foreign genes through lateral gene transfers. We hypothesised that the occurrence of these genomic islands within a *R. solanacearum* strain is related to the ability to cause potato brown rot disease and could be considered as pathogenicity islands (Hacker et al., 2004). However, the functions encoded by these specific genes could not be related to known pathogenic determinants. Among these brown rot strain specific genes, 37 (53%) encoded proteins with unknown functions, 6 encoded proteins involved in DNA synthesis and repair (helicase, thymidylate synthase, N-6 adenine-specific DNA methylase, DNA-binding protein, and part-B like nuclease), 2 encoded transmembrane proteins, 1 encoded a Tetratricopeptide repeat (TPR)-domain protein which is involved in protein-protein interactions, and 1 encoded a lipocalin described to play an important role in membrane biogenesis and repair and to be implicated in the dissemination of antibiotic resistance genes and in the activation of immunity (Bishop, 2000).

The DNA sequences identified in this study to be specific for potato brown rot strains of *R. solanacearum* are potentially useful for development of diagnostic assays. Interestingly, the potato brown rot strains specific primers 630 and 631 developed by Fegan et al. (1998) amplified a sequence of 357-bp between the IPO_02173 and IPO_02174 genes from the IPO1609 genome. This sequence is located not far from the extremity of the potato brown rot strain specific 29-gene cluster number 7 identified in the present work. This sequence was also detected in the sequenced potato brown rot

strain UW551 of *R. solanacearum* (Gabriel et al., 2006). The authors found that the sequence amplified by the 630-631 primers corresponded to the extremity of the potato brown rot strain specific 38-gene cluster RRSL02400-RRSL02437 of UW551. However, most genes of this specific cluster encode various bacteriophage proteins, which therefore have the potential to move from one bacterium to another. For this reason, detection methods based on this gene cluster may not be reliable in the long term. In the present work, 41% of the potato brown rot strain specific genes were parts of mobile genetic elements.

Some papers relate the utilization of mobile elements such as insertion sequences (IS) for specific detection of bacterial pathogens (Aittamaa et al., 2008). However, it is now well established that such elements can be deleted from the genome or can be transmitted from one strain to another by plasmid conjugation, DNA transformation or transduction by bacteriophages (Haagensen et al., 2002; Hacker et al., 2004). *R. solanacearum* is known to have a genome capable of rapid evolution because of its natural competence for transformation and the occurrence of horizontal gene transfers between strains (including the transfer of mobile genetic elements) (Bertolla et al., 1999; Fall et al., 2007; Salanoubat et al., 2002; Wicker et al., 2007). Thus, using a mobile element as marker for detection of particular *R. solanacearum* strains would decrease the reliability of the detection method by giving rise to false-positive or false-negative results.

From the list 70 specific genes for the potato brown rot IIB-1 strains identified in this study, 7 were not predicted to be part of mobile genetic elements or ACURs. Therefore, we further evaluate these genes as candidate targets for specific and reliable detection of IIB-1 strains. Finally we developed a multiplex PCR assay by combining a set of IIB-1 specific primers with universal primers for all *R. solanacearum* strains (Opina et al., 1997). The goal of this assay, designated as Mx-RsIIB1, is to provide a useful diagnostic tool for detection of all *R. solanacearum* strains, and specific identification of IIB-1 potato brown rot strains in the same PCR reaction. The primer pair 759-760 used in this multiplex reaction has been tested extensively and shown to be highly specific over a large number of *R. solanacearum* strains (Fegan and Prior, 2005; Opina et al., 1997). These primers amplified a 282-bp fragment located in

the upstream region of *R. solanacearum* UDP-3-O-acyl-GlcNAc deacetylase gene, which is highly conserved in this bacterium (Opina et al., 1997). The Mx-RsIIB1 assay also includes a set of primers targeting one of the IIB-1 specific genes identified in this study. These primers amplified a 329-bp fragment from the RSIPO_02103 gene, encoding a N-6 adenine-specific DNA methylase, supposed to be involved in DNA synthesis and repair. Specificity evaluation over 55 strains representative of the diversity found in the *R. solanacearum* species complex, confirmed the suitability of these primers for detection of potato brown rot strains belonging to the IIB-1 group. In addition primers were tested against a limited number of environmental isolates, and no cross reactions were detected. Nevertheless, further specificity evaluation should be done including a larger number of isolates obtained from different samples that can be subject for detection of *R. solanacearum* (e.g. potato tubers, soil, water courses).

To control bacterial wilt, the ability to detect the disease at a minimal level of infection and to eliminate infection sources and spread of the pathogen is essential. The availability of sensitive detection methods is of particular importance for control of potato brown rot strains, since IIB-1 strains are highly persistent in the environment and can cause symptomless latent infection in several hosts (Álvarez et al., 2008; Ciampi et al., 1980; Swanson et al., 2007; Van Elsas et al., 2000). The Mx-RsIIB1 assay developed in this study was capable to detect up to 50 pg of genomic DNA or 450 *R. solanacearum* cells per PCR reaction. These values are equivalent to sensitivity thresholds normally achieved by standard PCR methods previously developed for this pathogen (Elphinstone et al., 1996; Seal et al., 1993). However, the sensitivity significantly decreased when this method was applied for direct detection of *R. solanacearum* in artificially inoculated potato tuber extracts. The presence of inhibitory compounds in crude extracts of plant tissues or environmental samples is a major drawback of PCR detection protocols (Alvarez, 2005). Several approaches were applied to overcome PCR inhibition and results have been variable according to the nature of the sample (Poussier et al., 2002). In this study, the sensitivity of the Mx-RsIIB1 assay was improved by incorporating an enrichment step of the potato tuber extracts in a selective medium prior to the multiplex PCR reaction. This simple procedure, commonly called BIO-PCR, was previously proved to reduced the

concentration of inhibitory substances from a variety of environmental and plant samples, while increasing the number of target cells, thereby improving the detection sensitivity (Ozakman and Schaad, 2003; Pradhanang et al., 2000; Lin et al., 2009). However, the multiplex PCR assay developed in this study for specific detection of IIB-1 *R. solanacearum* strains has yet to be completely validated. Many variables remain to be optimized in order to achieve a higher sensitivity threshold for suitable detection of *R. solanacearum* in latently infected potato tubers, where the pathogen is present at very lows levels. Furthermore, this assay could also be evaluated for detection of *R. solanacearum* in other potential sources of pathogen inoculums like soil, waterways, or other plant hosts.

REFERENCES

Afshar B., Pitcher D., Nicholas R.A.J., and Miles R.J. 2008. An evaluation of PCR methods to detect strains of *Mycoplasma fermentans*. Biologicals 36:117-121.

Aittamaa M., Somervuo P., Pirhomen M., Mattinen L., Nissinen R., Auvinen P., Valkonen J.P.T. 2008. Distinguishing bacterial pathogens of potato using a genome-wide microarray approach. Mol. Plant Pathol. 9:705-717.

Alvarez A.M. 2005. Diversity and diagnosis of *Ralstonia solanacearum*. Pages 437- 447 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A.C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Álvarez B., López M.M., and Biosca E.G. 2008. Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. Microbiology. 154:3590–3598.

Bertolla F., Frostegard A., Brito B., Nesme X., and Simonet P. 1999. During infection of its hosts, the plant pathogen *Ralstonia solanacearum* naturally develops a state of competence and exchanges genetic material. Mol. Plant-Microbe Interact. 12:467-472.

Bishop R.E. 2000. The bacterial lipocalins. Biochimica and Biophysica Acta (BBA) – Prot. Struct. Mol. Enzymol. 1482:73-83.

Buddenhagen I., Sequeira L., and Kelman A. 1962. Designation of races in *Pseudomonas solanacearum*. Phytopathology 52:726.

Ciampi L., Sequeira L., and French E.R. 1980. Latent infection of potato tubers by *Pseudomonas solanacearum*. Am. Potato J. 57:377-386.

Cook D., and Sequeira L. 1994. Strain differentiation of *Pseudomonas solanacearum* by molecular genetic methods. Pages 77-94 in: Bacterial wilt- The disease and its causative agent, *Pseudomonas solanacearum.* A.C. Hayward, and G.L. Hartman, eds. CAB International, Wallingford, UK.

Elphinstone J.G. 2005. The current bacterial wilt situation: a global overview. Pages 9-28 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, and A.C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Elphinstone J.G., Hennessy J., Wilson J.K., and Stead D.E. 1996. Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. EPPO Bull. 26: 663–678.

Fall S., Mercier A., Bertolla F., Calteau A., Gueguen L., Perrière G., Vogel T.M., and Simonet P. 2007. Horizontal gene transfer regulation in bacteria as a “Spandrel” of DNA repair mechanisms. Plos One 2: e1055. doi:10.1371/journal.pone.0001055.

Fegan M., and Prior P. 2005. How complex is the "*Ralstonia solanacearum* species complex"? Pages 449-462 in: Bacterial Wilt: the Disease and the *Ralstonia solanacearum* species complex. C. Allen, P. Prior, and C. Hayward, eds. APS Press, St. Paul, MN, USA.

Fegan M., Holoway G., Hayward A.C., and Timmis J. 1998. Development of a diagnostic test based on the polymerase chain reaction (PCR) to identify strains of *R. solanacearum* exhibiting the biovar 2 genotype. Pages 35-43 in: Bacterial wilt disease. Molecular and ecological aspects. P. Prior, C. Allen, and J. Elphinstone, eds. Springer-Verlag, Berlin.

Fessehaie A., De Boer S.H., and Lévesque C.A. 2003. An oligonucleotide array for the identification and differentiation of bacteria pathogenic on potato. Phytopathology 93:262-269.

Gabriel D.W., Allen C., Schell M., Denny T.P., Greenberg J.T., Duan Y.P., Flores-Cruz Z., Huang Q., Clifford J.M., Presting G., González E.T., Reddy J., Elphinstone J., Swanson J., Yao J., Mulholland V., Liu L., Farmerie W., Patnaikuni M., Balogh B., Norman D., Alvarez A., Castillo J.A., Jones J., Saddler G., Walunas T., Zhukov A., and Mikhailova N. 2006. Identification of open reading frames unique to a select agent: *Ralstonia solanacearum* race 3 biovar 2. 1: Mol. Plant-Microbe Interact. 19:69-79.

Griep R.A., Van Twisk C., Van Beckhoven J.R.C.M., Van der Wolf J.M., and Schots A. 1998. Development of specific recombinant monoclonal antibodies against the lipopolysaccharide of *Ralstonia solanacearum*Ralstonia solanacearum race 3. Phytopathology 88**:**795-803

Guidot A., Prior P., Schoenfeld J., Carrere S., Genin S., and Boucher C.A. 2007. Genomic structure and phylogeny of the plant pathogen *Ralstonia solanacearum* inferred from gene distribution analysis. J. Bacteriol. 189:377-387.

Guidot A., Coupat B., Fall S., Prior P., and Bertolla F. 2009. Horizontal gene transfer between *Ralstonia solanacearum* strains detected by comparative genomic hybridization on microarrays. ISME J. 3:549–562.

Haagensen J.A.J*.,* Hansen S.K., Johansen T., and Molin S. 2002. In situ detection of horizontal transfer of mobile elements. FEMS Microbiol. Ecol. 42:261-268.

Hacker J., Hochhut B., Middendorf B., Schneider G., Buchrieser C., Gottschalk G., and Dobrindt U. 2004. Pathogenomics of mobile genetic elements of toxigenic bacteria. Int. J. Med. Microbiol. 293:453-461.

Hayward A.C. 1964. Characteristics of *Pseudomonas solanacearum*. J. Appl. Bacteriol. 27:265-277.

Hayward A.C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annu. Rev. Phytopathol. 29:65-87.

Hayward A.C. 1994a. Systematics and phylogeny of *Pseudomonas solanacearum* and related bacteria. Pages 123-135 in: Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A.C. Hayward and G.L. Hartman, eds. CAB International, Wallingford, UK.

Hayward A.C. 1994b. The hosts of *Pseudomonas solanacearum*. Pages 9-24 in: Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*. A.C. Hayward and G.L. Hartman, eds. CAB International, Wallingford, UK.

He L.Y., Sequeira L., and Kelman A. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant. Dis. 67:1357-1361.

Hendrick C., and Sequeira L. 1984. Lipopolysaccharide-defective mutants of the wilt pathogen *Pseudomonas solanacearum*. Appl. Environ. Microbiol. 48:94–101.

Janse J.D., Beld H.E.V.D., Elphinstone J., Simpkins S., Tjou-Tam-Sin N.A.A., and Vaerenbergh J.V. 2004. Introduction to Europe of *Ralstonia solanacearum* biovar 2, race 3 in *Pelargonium zonale* cuttings. J. Plant Pathol. 86:147-155.

Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693–695.

Kim S.H., Olson T.N., Schaad N.W., and Moorman G.W. 2003. *Ralstonia solanacearum* Race 3, biovar 2, the causal agent of brown rot of potato, identified in geranium in Pennsylvania, Delaware, and Connecticut. Plant Dis. 87:450.

Lambert C.D. 2002. Agricultural bioterrorism protection act of 2002: Possession, use, and transfer of biological; agents and toxins; interim and final rule. (7 CFR Part 331). Federal Register 67:76908-76938.

Lin C.-H., Hsu S.-T., Tzeng K.-C., and J.-F. Wang. 2009. Detection of race 1 strains of *Ralstonia solanacearum* in field samples in Taiwan using a BIO-PCR method. Eur. J. Plant Pathol. 124:75–85.

Occhialini A., Cunnac S., Reymond N., Genin S., Boucher C. 2005. Genome-wide analysis of gene expression in *Ralstonia solanacearum* reveals that the hrpB gene acts as a regulatory switch controlling multiple virulence pathways. Mol. Plant-Microbe Interact. 18:938-49.

Opina N., Tavner F., Hollway G., Wang J.-F., Li T.-H., Maghirang R., Fegan M., Hayward, A.C., Krishnapillai V., Hong W.-F., Holloway B.W., and Timmis J.N. 1997.A novel method for development of species and strain specific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). Asia-Pac J. Mol. Biol. 5:19-30.

Ozakman, M., and N.W. Schaad. 2003. A real-time BIO-PCR assay for detection of *Ralstonia solanacearum* race 3, biovar 2, in asympotamtic potato tubers. Can. J. Plant Pathol. 25:232-239.

Pegg K., and Moffett M. 1971. Host range of the ginger strain *of Pseudomonas solanacearum* in Queensland. Aust. J. Exp. Agric. Anim. Husb. 11:696-698.

Poussier S., Cheron J-J, Couteau A., and Luisetti J. 2002. Evaluation of procedures for reliable PCR detection of *Ralstonia solanacearum* in common natural substrates. J. Microbiol. Meth. 51:349-359.

Pradhanang P.M., Elphinstone J.G. and R.T.V. Fox. 2000. Sensitive detection of *Ralstonia solanacearum* in soil: a comparison of different detection techniques. Plant Pathol. 49: 414-422.

Prior P., and Fegan M. 2005. Recent development in the phylogeny and classification of *Ralstonia solanacearum*. Acta Hort. 695:127-136.

Priou S., Gutarra L., and Alley P. 1999. Highly sensitive detection of *Ralstonia solanacearum* in latently infected potato tubers by postenrichment enzyme-linked immunosorbent assay on nitrocellulose membrane. EPPO Bull. 29:117-125

Reymond N., Charles H., Duret L., Calevro F., Beslon G., and Fayard J.M. 2004. ROSO: optimizing oligonucleotide probes for microarrays. Bioinformatics. 20:271-273.

Salanoubat M., Genin S., Artiguenave F., Gouzy J., Mangenot S., Arlat M., Billault A., Brottier P., Camus J.C., Cattolico L., Chandler M., Choisne N., Claudel-Renard C., Cunnac S., Demange N., Gaspin C., Lavie M., Moisan A., Robert C., Saurin W., Schiex T., Siguier P., Thebault P., Whalen M., Wincker P., Levy M., Weissenbach J. and Boucher C.A. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415:497-502.

Sanchez Perez A., Mejia L., Fegan M., and Allen C. 2008. Diversity and distribution of *Ralstonia solanacearum* strains in Guatemala and rare occurrence of tomato fruit infection. Plant Pathol. 57:320-331.

Seal S.E., Jackson L.A., Young J.P.W., and Daniels M.J. 1993. Differentiation of *P. solanacearum, P. syzygii, P. pickettii* and the blood disease bacterium by partial 16 S rRNA sequencing: construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. J. Gen. Microbiol. 139:1587–1594.

Swanson J.K., Montes L., Mejia L., and Allen C. 2007. Detection of latent infections of *Ralstonia solanacearum* race 3 biovar 2 in geranium. Plant Dis. 91:828-834.

Tambong J.T., de Cock A.W.A.M., Tinker N.A., and Lévesque C.A. 2006. Oligonucleotide array for identification and detection of *Pythium* species. Appl. Environ. Microbiol. 72:2691-2706.

Terol J., Munoz-Soriano V., Arahal D.R., Buades C., Pérez-Pérez A., Llop P., Belmonte U. C.F., López M.M., and Pérez-Alonso M. 2006. Random genome sequencing of *Ralstonia solanacearum* strain IVIA 1602 and comparative analysis with strain GMI1000. J. Phytopathol. 154:556-564.

Untergasser A., Nijveen H., Rao X., Bisseling T., Geurts R., and Leunissen J.A.M. 2007. Primer3Plus, an enhanced web interface to Primer3. Nucl. Ac. Res. 35:W71-W74; doi:10.1093/nar/gkm306.

USDA-United States Department of Agriculture. 2004. *Ralstonia solanacearum* race 3 biovar 2. Southern Geranium. New Pest Response Guidelines. USDA, APHIS, PPQ. Riverdale, MD.

Van Elsas J.D., Kastelein P., Van Bekkum P., Van der Wolf J.M., De Vries P.M., Van Overbeek L.S. 2000. Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathology 90:1358–1366.

Weller S.A., Elphinstone J.G., Smith N.C., Boonham N., and Stead D.E. 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Appl. Environ. Microbiol. 66:2853-2858.

Wicker E., Grassart L., Coranson-Beaudu R., Mian D., Guilbaud C., Fegan M., and Prior P. 2007. *Ralstonia solanacearum* strains in Martinique (French West Indies) exhibiting a new pathogenic potential. Appl. Environ. Microbiol. 73:6790-6801.

CAPÍTULO 5

DISCUSIÓN GENERAL Y PERSPECTIVAS

DISCUSIÓN GENERAL

Ralstonia solanacearum es una bacteria fitopatógena muy compleja y de difícil control. El propósito de esta tesis consistió en generar conocimiento sobre el tipo de cepas de *R. solanacearum* que afectan los cultivos de papa en Uruguay, como prerrequisito fundamental para el desarrollo e implementación de estrategias de control eficientes. A continuación se resumen los principales resultados obtenidos y se discuten sus implicancias en lo que refiere al control de la enfermedad.

Según el relevamiento realizado (Capítulo 2), todas las cepas de *R. solanacearum* que afectan a los cultivos de papa en Uruguay pertenecen al filotipo IIB secuevar 1. Este tipo de cepas es el que generalmente predomina en climas fríos y templados (Elphinstone, 2005). El hecho de que los cultivos estén afectados por un mismo tipo de cepas con poca variabilidad genética, resulta alentador en lo que refiere a las posibilidades de lograr un control efectivo de la enfermedad a través del uso de variedades de papa resistentes. Se ha reportado la dificultad de introducir resistencia estable a la marchitez bacteriana en cultivares de papa (López y Biosca, 2005). Esto se debe principalmente a la variabilidad de respuesta observada en localidades con condiciones ambientales diferentes, o frente a distintas variantes del patógeno (French y De Lindo, 1982; Anguiz y Mendoza, 1997). Estas experiencias resaltan la necesidad de contar con variedades bien adaptadas a las condiciones de producción del cultivo y con resistencia específica frente al tipo de cepas presentes en el país. En Uruguay, se cuenta con la especie silvestre *S. commersonii* como principal fuente de germoplasma para la introducción de resistencia en el programa de mejoramiento genético de papa. En base a esto, se incluyó a esta especie en el análisis comparativo sobre la agresividad de las cepas uruguayas de *R. solanacearum.* Los resultados obtenidos demostraron la existencia de diferentes niveles de resistencia en *S. commersonii*, y contribuyeron a la selección de los genotipos como progenitores para los cruzamientos. Por otro lado, este estudio reveló diferencias significativas en la agresividad de las cepas uruguayas de *R. solanacearum*. Este hallazgo, resalta la necesidad de utilizar las cepas más agresivas en las etapas de tamizaje y selección de genotipos resistentes en el programa de mejoramiento.

El haber detectado diferencias de agresividad entre las cepas de *R. solanacearum*, también ofrece la posibilidad de analizar las razones que expliquen este comportamiento y profundizar en los mecanismos de virulencia de este patógeno (Capítulo 3). Para ello, se realizó un estudio genómico comparativo basado en el uso de un chip de microarrays que representa el pan-genoma de *R. solanacearum.* Este chip fue desarrollado por C. Boucher y colaboradores (INRA-CNRS, Toulouse, Francia) a partir de la secuencia completa de 3 cepas diferentes de *R. solanacearum,* incluyendo a la cepa IPO1609 perteneciente al grupo IIB-1. El uso de microarrays para la realización de comparaciones genómicas a gran escala de cepas de *R. solanacearum* fue previamente validado (Guidot et al., 2007). En esta tesis, esta herramienta demostró ser lo suficientemente poderosa como para detectar diferencias genéticas incluso entre cepas muy relacionadas. Los resultados de este estudio permitieron establecer el repertorio genético de cada cepa analizada e identificar una lista de genes candidatos de virulencia potencialmente involucrados en las diferencias de agresividad entre cepas. En esta lista se encontró un grupo de genes que codifican para proteínas relacionadas con las fimbrias de tipo IV. La decisión de profundizar en la actividad biológica de estas proteínas estuvo basada en varias razones: i) las fimbrias de tipo IV median una variedad de procesos celulares y son considerados importantes factores de virulencia en muchas bacterias patógenas (Pizzarro-Cerdá y Cossart, 2006); ii) en *R. solanacearum,* se ha comprobado que estas estructuras están involucradas en la virulencia, aunque los estudios realizados hasta el momento son limitados ya que abarcan pocas cepas y se basan en el uso de tomate como planta hospedera (Kang et al., 2002; Liu et al., 2001); iii) en el genoma de *R. solanacearum* existen aproximadamente 40 genes que presuntamente están involucrados en la biogénesis y función de fimbrias de tipo IV, pero actualmente solo se ha explorado la función de algunos de estos genes y sus roles en la virulencia todavía no han sido dilucidados (Kang et al., 2002; Liu et al., 2001); iv) según analogía respecto a otros microorganismos patógenos, los genes identificados en este estudio, codifican para un grupo de proteínas denominadas pilinas menores, las cuales se ha comprobado que están involucradas en la biogénesis y función de fimbrias de tipo IV (Koomey, 1995; Helain et al., 2007; Giltner et al., 2010). Los estudios preliminares realizados en el

marco de esta tesis, sugieren que estas proteínas tienen un efecto directo sobre la agresividad y la capacidad de colonización de *R. solanacearum* en plantas de papa. Sin embargo, los resultados obtenidos son parciales y consideramos que este estudio sólo significa un primer abordaje al tema, que nos permitirá generar nuevas hipótesis de trabajo para seguir profundizando sobre el rol de estas proteínas en la virulencia de *R. solanacearum.*

La información generada a partir del estudio genómico comparativo de cepas uruguayas de *R. solanacearum* por CGH también se utilizó para realizar comparaciones genómicas respecto a otras cepas dentro de este complejo de especies (Capítulo 4). Este análisis permitió identificar una lista de secuencias específicas presentes en cepas causantes de la marchitez bacteriana de la papa, pertenecientes al grupo IIB-1 (Guidot et al., 2009). La mayoría de estas secuencias se encuentran agrupadas en islas genómicas probablemente adquiridas por mecanismos de transferencia genética horizontal. Las secuencias identificadas en este estudio constituyen potenciales blancos de detección de cepas de *R. solanacearum* IIB-1. En particular, en esta tesis se seleccionó uno de estos genes específicos para el desarrollo y optimización de un método basado en PCR aplicable a la detección del patógeno en muestras de tubérculos-semilla. Se ha comprobado que este tipo de cepas, tienen una alta capacidad de persistencia en el ambiente así como de producir infecciones latentes en material vegetativo lo que favorece su diseminación y propagación (Álvarez et al., 2008; Elphinstone et al., 1998; Swanson et al., 2005; Van Elsas et al., 2000; Williamson et al., 2002). Debido a estas características, resulta fundamental contar con métodos de diagnóstico eficientes que contribuyan a evitar la disminación del patógeno y asistan al control integrado de la enfermedad.

PERSPECTIVAS

Esta tesis permitió generar información sobre el tipo de cepas de *R. solanacearum* que afectan los cultivos de papa en Uruguay. Sin embargo, como sucede en la mayoría de las investigaciones, los avances alcanzados generan nuevas preguntas que quedan por responder. A continuación se describen las perspectivas que surgen a partir de la información y experiencia generadas en estos años de trabajo, las cuales espero continuar desarrollando en el futuro en el marco de nuevas investigaciones que involucren la participación de todos los actores involucrados en esta problemática.

Una de las perspectivas naturales que surgen de este estudio es la necesidad de continuar con el relevamiento de cepas de *R. solanacearum* presentes en el país, abarcando también otros hospederos susceptibles o nichos ecológicos que pueden albergar al patógeno. Este relevamiento, también debe estar dirigido a fortalecer la vigilancia epidemiológica por parte de los organismos de control gubernamentales respecto al riesgo de introducción de otras variantes del patógeno, en particular, a través de zonas limítrofes con Brasil donde se ha reportado la presencia de cepas de *R. solanacearum* raza 1.

La implementación de métodos que contribuyan al diagnóstico precoz de *R. solanacearum* juega un papel fundamental en varias de las medidas que involucra el control integrado de la enfermedad. Uno de los aspectos sobre los cuales se prevé seguir trabajando es en la optimización de métodos moleculares para la detección de *R. solanacearum* en lotes de tubérculos-semilla, ya que constituyen una de las principales vías de diseminación del patógeno. En particular, se plantea terminar de optimizar el ensayo Mx-RsIIB1 y evaluar blancos de detección adicionales identificados en este estudio. Esta tecnología se transferirá al Laboratorio de Bacteriología de la Dirección General de Servicios Agrícolas (DGSA), de forma de lograr la incorporación de métodos moleculares de detección a los protocolos de control fitosanitario de *R. solancearum*.

La introducción de resistencia a *R. solanacearum* en nuevas variedades de papa en el marco del programa de mejoramiento que se desarrolla en INIA, constituye la

estrategia mas efectiva para alcanzar el control de la enfermedad a largo plazo. Tradicionalmente, la selección de plantas con resistencia a este patógeno está basada en la ausencia de signos de marchitamiento en ensayos de inoculación. Sin embargo, desde hace unos años se ha confirmado la presencia de infección latente en tallos y tubérculos de clones mejorados con cierto grado de resistencia a *R. solanacearum* (Priou et al., 2001, Priou et al., 2005). Estos hallazgos revelaron la necesidad de implementar criterios más exigentes de selección que incluyan la detección de infección latente en el material resultante de los programas de mejoramiento, de forma de evitar la generación de variedades tolerantes que promuevan la diseminación de la enfermedad en condiciones climáticas predisponentes. En base a esto, una de las perspectivas en las cuales se proyecta seguir trabajando, es en la optimización y aplicación de nuevos métodos que permitan detectar la presencia del patógeno en material vegetal de forma de asistir al programa de mejoramiento genético en la evaluación y selección de genotipos con resistencia verdadera frente a *R. solanacearum.* En este sentido, se proyecta trabajar con dos enfoques diferentes. Por un lado, se plantea la aplicación de métodos de detección molecular basados en PCR, que presenten una adecuada sensibilidad como para detectar la presencia de infección latente en tallos y tubérculos de clones que no muestran síntomas de marchitamiento por *R. solanacearum.* Además, se propone desarrollar un método de *screening* de germoplasma basado en el uso de una cepa de *R. solanacearum* reportera marcada con la proteína verde fluorescente. Esta herramienta, representa una alternativa simple y versátil para el monitoreo del patógeno *in vivo* durante el proceso de infección. Lo que se pretende es incorporar esta tecnología para realizar un estudio comparativo de la capacidad de colonización, diseminación y multiplicación de este patógeno, sobre el material disponible en el programa de mejoramiento. De esta forma se pretende contribuir al mejor aprovechamiento del germoplasma existente, así como a profundizar en el estudio de los mecanismos que explican la resistencia observada en *S. commersonii.*

Por último, se plantea seguir trabajando en el estudio de los mecanismos de virulencia de *R. solanacearum,* tomando como punto de partida la información generada en este estudio. Se completará el análisis funcional de los genes relacionados con las fimbrias

de tipo IV para determinar cual es su rol en la virulencia de *R. solanacearum*. Las perspectivas propuestas en este tema están detalladas en el Capítulo 4. En particular, se plantea evaluar otras actividades mediadas por estas estructuras superficiales omo ser la formación de biofilm, la autoagregación y la adherencia a superficies. Estos análisis nos permitirán investigar el mecanismo por el que estas proteínas actúan y comprender mejor sus implicancias sobre la virulencia de *R. solanacearum.* También se evaluará el rol de cada proteína de forma individual y se completará el análisis de los mutantes con ensayos de complementación. Además se iniciarán estudios sobre otros genes candidatos identificados, en particular sobre las proteínas efectoras secretadas por el sistema de secreción de tipo III, debido al rol central que presenta este sistema en la patogenia de *R. solanacearum.* Con estos estudios se pretende avanzar en la comprensión de los mecanismos a través de los cuales *R. solanacearum* interacciona con su hospedero provocando la enfermedad. Hasta el momento, la mayoría de los estudios sobre la virulencia de *R. solanacearum* están centrados en tomate o en la planta modelo *Arabidopsis,* y existe poca información sobre lo que ocurre en otros hospederos. En base a esto, se propone trabajar sobre papa como hospedero natural del tipo de cepas IIB-1, y así generar conocimiento sobre este patosistema. Es de esperar que estos conocimientos sean de utilidad para el desarrollo de nuevas estrategias de control para este importante patógeno.

REFERENCIAS

Álvarez B., López M.M., y Biosca E.G. 2008. Survival strategies and pathogenicity of *Ralstonia solanacearum* phylotype II subjected to prolonged starvation in environmental water microcosms. Microbiology. 154:3590–3598.

Anguiz R.J. y Mendoza H.A. 1997. General and specific combining abilities for resistance to bacterial wilt (*Pseudomonas solanacearum* E.F. Smith) in PVX and PVY immune autotetraploid potatoes. Fitopatologia. 32: 71-80.

Boshou L. 2005. A broad review and perspective on breeding for resistance to bacterial wilt. Pages 225-238 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A. C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Elphinstone J.G. 2005. The current bacterial wilt situation: a global overview. Pages 9-28 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A.C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Elphinstone J.G., Stanford H.M., y Stead D.E. 1998. Detection of *Ralstonia solanacearum* in potato tubers, *Solanum dulcamara*, and associated irrigation water. Pages 133–139 in: Bacterial Wilt Disease: Molecular and Ecological Aspects. P. Prior, C. Allen, y J. Elphinstone, eds. Springer-Verlag, Berlin.

French E.R. y De Lindo L. 1982. Resistance to *Pseudomonas solanacearum* in potato: specificity and temperature sensitivity. Phytopathology. 72:1408-1412.

Giltner C.L., Habash M., y Burrows L.L. 2010. *Pseudomonas aeruginosa* minor pilins are incorporated into type IV pili. J. Mol. Biol. 398: 444–461.

Guidot A., Prior P., Schoenfeld J., Carrere S., Genin S., y Boucher C.A. 2007. Genomic structure and phylogeny of the plant pathogen *Ralstonia solanacearum* inferred from gene distribution analysis. J. Bacteriol. 189:377-387.

Guidot A., Elbaz M., Carrere S., Siri M.I., Pianzzola M.J., Prior P. y Boucher C. 2009. Specific genes from the potato brown rot strains of *Ralstonia solanacearum* and their potential use for strain detection. Phytopathology 99:1105–1112.

Helaine S., Dyer D.H., Nassif X., Pelicic V., y Forest K.T. 2007. 3D structure/function analysis of PilX reveals how minor pilins can modulate the virulence properties of type IV pili. Proc. Natl. Acad. Sci. USA 104: 15888–15893.

Kang Y., Liu H., Genin S., Schell M.A., y Denny T.P. 2002. *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. Mol. Microbiol. 46:427-437.

Koomey M. 1995. Prepilin-like molecules in type 4 pilus biogenesis: minor subunits, chaperones or mediators of organelle translocation? Trends Microbiol. 3(11):409-10.

Liu H., Kang Y., Genin S., Schell M.A., y Denny T.P. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiology 147:3215-3229.

López M.M. y Biosca E.G. 2005. Potato bacterial wilt management: new prospects for an old problem. Pages 205-224 in: Bacterial Wilt: The Disease and the *Ralstonia*

solanacearum Species Complex. C. Allen, P. Prior, y A.C. Hayward, eds. American Phytopathological Society, St.Paul, MN.

Pizarro-Cerdá J. y Cossart P. 2006. Bacterial adhesion and entry into host cells. Cell 124:715–727.

Priou S., Torres R., Villar A., Gutarra L., y De Mendiburu F. 2001. Optimization of sample size for the detection of latent infection by *Ralstonia solanacearum* in potato seed tubers in the highlands of Peru. Pot. Res. 44:349–58.

Priou S., Aley P., y Gutarra L. 2005. Assessment of resistance to bacterial wilt in CIP advanced potato clones. Pages 261-267 in: Bacterial Wilt: The Disease and the *Ralstonia solanacearum* Species Complex. C. Allen, P. Prior, y A.C. Hayward, eds. American Phytopathological Society, St. Paul, MN.

Swanson J.K., Yao J., Tans-Kersten J.K., y Allen C. 2005. Behavior of *Ralstonia solanacearum* race 3 biovar 2 during latent active infection of geranium. Phytopathology 95:136-143.

Van Elsas J.D., Kastelein P., Van Bekkum P., Van der Wolf J.M., De Vries P.M., Van Overbeek L.S. 2000. Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. Phytopathology 90:1358–1366.

Williamson L., Nakaho K., Hudelson B., y Allen C. 2002. *Ralstonia solanacearum* race 3, biovar 2 strains isolated from geranium are pathogenic on potato. Plant Dis. 86:987-991.