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CHARACTERIZATION AND MECHANISM OF ACTION OF THE BIOLOGICAL CONTROL *Pantoea agglomerans* EPS125

Ma Carmen MORENO GONZÁLEZ

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Doctoral Thesis

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Characterization and mechanism of action of the biological control agent *Pantoea agglomerans* EPS125

Memòria presentada per Mª Carmen Moreno González, inscrit en el programa de doctorat de Ciències per optar al Grau de Doctor per la Universitat de Girona

Mª Carmen Moreno González

Emili Montesinos Seguí i Esther Badosa Romanyó, Catedràtic i Professora de l'Àrea de Patologia Vegetal respectivament, membres del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària i del Institut de Tecnologia Agroalimentària de la Universitat de Girona,

CERTIFIQUEN

Que la llicenciada en Biologia Mª Carmen Moreno González ha dut a terme, sota la seva direcció, el treball amb el títol "*Characterization and mechanism of action of the biological control agent Pantoea agglomerans EPS125"*, que presenta en aquesta memòria la qual constitueix la seva Tesi per a optar a Grau de Doctor per la Universitat de Girona.

I per a què consti als efectes oportuns, signen la present a Girona, 8 de juny del 2006.

Vist-i-plau

Dr. Emili Montesinos Seguí

Dra. Esther Badosa Romanyó

Emili Montesinos Seguí, catedràtic de l'àrea de Patologia Vegetal del Departament d'Enginyeria Química, Agrària i Tecnologia Agroalimentària de la Universitat de Girona, i director dels projectes de recerca 'Optimización de los métodos de detección, prevención y control de fuego bacteriano de las rosáceas en España' (Ref. AGL2001-2349-C03-01) i 'Evaluación de un procedimiento de bioprotección de fruta y hortalizas frescas para el control de podredumbres fúngicas y de patógenos causantes de toxiinfecciones alimentarias' (Ref. CAL03-084)

MªCarmen Moreno González que ha inscrit la tesi doctoral titulada *"Characterization and mechanism of action of the biological control agent Pantoea agglomerans EPS125"* amb el número de registre 613 (28 d'octubre del 2003)

DECLAREN

Que aquesta tesi forma part dels projectes de recerca anteriorment citats i que està sotmesa a la propietat intel·lectual compartida amb els investigadors del grup de Patologia de la Universitat de Girona que participen en els esmentats projectes (Article 2. apartat 2, RD 1326/2003 de 24-10-2003; Llei de la Propietat Intel·lectual, RD 1/1996 de 12-04-1996).

I per a què consti als efectes oportuns, signen la present a Girona el 14 de juliol de 2006.

Vist-i-plau

Dr. Emili Montesinos Seguí

MªCarmen Moreno González

A la memoria de mi yayo

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Summary

In recent years, an exhaustive screening of biological control agent (BCA) was performed in our laboratory obtaining bacterial isolate EPS125 from pear fruit surface, being highly effective against *Penicillium expansum,* one of the most important fungal pathogen in postharvest diseases. This strain also has shown effectiveness against a wide range of fungal pathogens including *Botrytis cinerea, Monilinia laxa* and *Rhizopus stolonifer* and in a large variety of fruit. However, to develop this strain as commercial biopesticide, an extensive characterization including identification, effectiveness, mass production, formulation, biocontrol mechanism and monitoring studies is essential. So far, mass production and formulation processes have been successfully developed for strain EPS125; as well as an initial approach to its biocontrol mechanism has been performed, in which preemptive exclusion by wound colonization and direct interaction with pathogen spores seem to be involved. Nowadays, strain EPS125 is deposited in the Spanish Type Culture Collection as *Pantoea agglomerans* CECT5392 and is protected under a patent covering its use as biofungicide in several European countries.

The main objective of this PhD thesis was to complete the necessary information for the future registration of this biopesticide including (i) identification and characterization of strain EPS125 through phenotypic and genotypic methods; (ii) development of a monitoring method for strain EPS125 through specific DNA molecular markers; (iii) determination of its biocontrol mechanism against *P. expansum* by means of phenotypic approaches and genotypic studies.

According to findings obtained from morphological and biochemical tests, API 20E, Biolog and fatty acid methyl ester profiles and 16S rDNA gene sequence; strain EPS125 was included in *Pantoea agglomerans* (*Enterobacter agglomerans-Erwinia herbicola*) species. This strain showed typical traits different from other bacteria including not only strains from different genus, but also strains of *Enterobacter-Erwinia-Pantoea* group. For example strain EPS125 is unable to use tween 40 as carbon source and is able to metabolize mono-methyl succynate contrarily to all analysed strains; as well as it is the only strain containing 14:1 w5c, SIF1 and 15:0 ISO 3OH fatty acids. In addition, *P. agglomerans* EPS125 shows a characteristic macrorestriction fragment length polymorphism (MRFLP) pattern of digestion of DNA with *XbaI* of 13 fragments (302, 270, 259, 253, 197, 179, 165, 160, 140, 110, 81 and 71 Kb).

Two different DNA molecular markers of *P. agglomerans* EPS125, named 125.2 and 125.3, have been obtained in the present work. Each molecular marker was semispecific in the detection of strain EPS125 by means of PCR reaction when they were used separately, amplifying five additional *E. herbicola* strains with the 125.2 primer set and one *E. herbicola* strain in case of the 125.3 primer set from an overall of 267 strains (257 *E. herbicola* strains and 10 strains of different genera). Nevertheless, only strain EPS125 showed amplification signal with both molecular

markers suggesting that the combined use of the two primer sets is suitable for the specific detection of *P. agglomerans* EPS125 in a multiplex PCR reaction. Nowadays, a real time PCR using the 125.3 design has been successful for strain EPS125; however, a monitoring method based on real time PCR combining 125.2 and 125.3 molecular markers marked with different reporter dye have not been developed yet.

P. agglomerans EPS125 contains essential features required for a commercial biopesticide, that is, it did not present ice nucleation activity causing frost damage on plants, and it is not a plant pathogen according to the absence of hypersensitive response on tobacco plants. In addition, *P. agglomerans* EPS125 is neither pathogen nor toxic for animals.

The effectiveness of the biocontrol agent *P. agglomerans* EPS125 to inhibit *P. expansum* was determined by means of dose-response assays, which provided efficiency parameters. Specifically, strain EPS125 was highly effective against *P. expansum* in apple fruit showing a median effective dose from $2.7x10^5$ to $7x10^5$ cfu/ml and a ratio of 25-101 EPS125 cells required to inactivate one pathogen spore according to the hyperbolic saturation model.

Biocontrol mechanism used by *P. agglomerans* EPS15 to control *P. expansum* was studied by means of phenotypic approaches as well as through mutagenesis with transposons. According to phenotypic data, antibiosis and competition for nutrients are not responsible of biocontrol activity of strain EPS125. On the contrary, direct interaction arises as an important biocontrol mechanism as cell-to-cell contact between biocontrol and pathogen was needed to achieve inhibition of conidia germination.

Observations performed with scanning and transmission electron microscopes on cultures grown in apple juice showed that this strain produces abundant pili-like structures (PLS) interconnecting cells similar to type IV pili, and forms multicellular aggregate structures or symplasmata surrounded by an alginate saccharic layer. Nevertheless, these cellular aggregations were not observed in EPS125 cultures grown in LB broth. All seems to point out that alginate, which is produced under certain growth conditions, and PLS that may be implied in twitching movement of EPS125 cells and in the formation of microcolonies, might be involved in the early steps of biofilm formation of *P. agglomerans* EPS125 and in the inhibition of pathogen spore germination.

To demonstrate what mechanism is responsible of the biocontrol activity of *P. agglomerans* EPS125, genetic analysis by means of random mutagenesis with minitransposon GUS (mTn5SS*gusA40*) was performed. A collection of 4032 non auxotrophic mutants was submitted to screening and 7 mutants (m40, m439, m622, m1210, m2002, m2126 and m4015) completely defective in biocontrol of *P. expansum* infections in apple fruit were chosen. All the selected mutants showed the same colony morphology on ABM minimal medium; growth potential in LB broth; growth on apple fruit at 13 and 23 ºC; production of signal molecules of quorum sensing; and qualitative production of alginate. However, interesting phenotypic traits only for m2126 and m4015 mutants were observed by using electronic microscopy. Genetic analysis only was accomplished for m40, m2002, m2126 and m4015 mutants.

The genomic sequence flanked to minitransposon in m40 mutant was sequenced and the expected protein obtained *in silico* showed a 35% of identity with dihydrodipicolinate synthases (DHDPS) of several bacteria. DHDPS is a key enzyme of the biosynthetic pathway of *meso*-diaminopimelate (*meso*-DAP) in bacteria and plants, which is the direct precursor of lysine and an essential constituent of the bacterial peptidoglycan layer. Due to the fact m40 mutant was not lysine auxotroph and had the biosynthetic way of DAP unaltered being able to grow in minimal defined media; it had three transposon insertions into its genome according to southern analysis; and the DNA sequence flanking to transposon did not show similarity with any DNA sequence deposited in the GenBank database, place this mutant as an unsuitable candidate to be consider for further studies.

In mutant m2126, the hypothetical proteins deduced from nucleotide sequence of the transposon flanking region shared similarity with a hypothetical protein with unknown function of *Comamonas* sp. (33 %) and with bacterial luciferase family proteins of several microorganisms (55 %). It may be that m2126 mutant has damaged a regulation system via quorum sensing affecting the normal production of PLS structures and the consequent biofilm formation.

Mutant m4015 showed 41 % of identity with lysine/ornithine N-monooxygenases which are implied in aerobactin siderophore biosynthesis. This mutation may alter the ability to acquire external iron, resting large amounts available for *P. expansum* which is needed for germination of *P. expansum* spores. In addition, iron limitation may alter the production of PLS producing the abnormal biofilm showed by m4015 mutant.

Mutants m2002 and m4015 showed 90 % of identity with IS1222 insertion sequences, which contribute to genetic flexibility and environmental adaptation of microorganisms through their rearrangements or deletions. Although both sequences were identical, it is likely that transposon was inserted in different points of genomic DNA of the two mutants due to their southern patterns and phenotypic features were not the same. Therefore, *P. agglomerans* EPS125 may have several IS1222 copies into its genome.

Evidences obtained in this work allow the development of a hypothetical model for biofilm formation of *P. agglomerans* EPS125, wherein inhibition of germination of pathogen spores is involved. Firstly, antagonist cells may attach to the apple surface using a variety of cell surface components such as PLS and alginate. PLS can bind to a variety of surfaces including inert surfaces, other bacterial cells and pathogen spores. These structures bring cells to form cellular clusters or microcolonies, in which pathogen conidia are included. Similarly, initial adhesion on apple surface is followed by the production of extracellular polysaccharide alginate, which only is induced under certain growth conditions. Moreover, EPS125 cells may be able to take up iron from apple fruit by means of siderophores slowing down iron availability to pathogen cells, and avoiding germination process of *P. expansum*. Therefore, the microenvironment formed by strain EPS125 may bring down the availability of compounds such as water, iron or oxygen required for germination of *P. expansum* conidia. In addition, strain EPS125 produces HSL molecules, which are implied in the regulation via quorum sensing of specific target genes that may be responsible of biofilm differentiation and biocontrol activity. Specifically, it may control the formation of PLS, which are an important factor for the normal development of biofilms. In summary, biofilm formed by *P. agglomerans* EPS125 provides an appropriate milieu for its growth and a physical structure that allows to withstand environmental stresses, as well as it may inhibit *P. expansum* infection depriving spores of essential compounds such as water, oxygen or iron which trigger the germination process, thus justifying the need for cell-to-cell interaction.

In conclusion, results obtained in the present work extend the knowledge about the behaviour of *P. agglomerans* EPS125 on its natural environment and reinforce this strain as an interesting biopesticide for commercial development.

Resumen

En los últimos años, nuestro grupo de investigación ha realizado varias prospecciones en búsqueda de nuevos agentes de biocontrol efectivos en el control de enfermedades de poscosecha. En una de ellas se aisló la cepa EPS125 que mostró elevada eficacia de biocontrol del patógeno fúngico *Penicillium expansum*, causante de la podredumbre azul y de grandes pérdidas económicas en poscosecha de fruta. Esta cepa también mostró ser efectiva frente a un amplio espectro de patógenos fúngicos de poscosecha como *Botrytis cinerea, Monilinia laxa* y *Rhizopus stolonifer* y en una gran variedad de frutos. Debido a su alta eficacia, se planteó desarrollar esta cepa comercialmente; no obstante, para poder lograr este objetivo se debe llevar a cabo una exhaustiva caracterización del producto a nivel de identificación, producción en masa, formulación, mecanismos de biocontrol y trazabilidad. Hasta el momento, aspectos como la producción en masa y formulación de la cepa EPS125 han sido desarrollados con éxito. También, aproximaciones sobre el mecanismo de biocontrol de dicha cepa han sido realizadas, en donde la exclusión preventiva del patógeno por colonización del nicho y la interacción directa con las esporas fúngicas y tubos germinativos parecen tener un papel clave en el biocontrol.

En el presente trabajo se planteó como objetivo complementar la información necesaria para el futuro registro del biopesticida EPS125, el cuál se puede desglosar en (i) identificación y caracterización de la cepa EPS125 a través de pruebas fenotípicas y genotípicas; (ii) desarrollo de un método de trazabilidad para la cepa EPS125 a través de marcadores moleculares específicos; y (iii) determinación del mecanismo de biocontrol utilizado por la cepa EPS125 contra *P. expansum* a través de aproximaciones fenotípicas y estudios genotípicos.

De acuerdo con los resultados obtenidos en las pruebas morfológicas y bioquímicas, API 20E, perfiles Biolog y de ácidos grasos, y secuenciación del gen 16S rDNA, la cepa EPS125 se incluye dentro de la especie *Pantoea agglomerans* (*Enterobacter agglomerans-Erwinia herbicola*). Esta cepa mostró rasgos característicos que la diferenciaban de otras especies, e incluso de cepas de la misma especie. En concreto, a diferencia del resto de cepas analizadas, la cepa EPS125 fue incapaz de utilizar como fuente de carbono tween 40, pero por el contrario fue la única capaz de metabolizar mono-metil succinato. Además, en relación al perfil de ácidos grasos celulares mostrados por la cepa EPS125, ésta también fue la única que mostró los ácidos grasos 14:1 w5c, SIF1 y 15:0 ISO 3OH. En relación con la caracterización genotípica, la cepa EPS125 mostró un polimorfismo en la longitud de los fragmentos de macrorestricción genómica (MRFLP) peculiar compuesto por 13 fragmentos (302, 270, 259, 253, 197, 179, 165, 160, 140, 110, 81 y 71 Kb) a partir de la digestión del DNA con *XbaI*.

Dos marcadores moleculares de DNA específicos (125.2 y 125.3) para la cepa EPS125 han sido desarrollados en el presente trabajo. Cada marcador molecular mostró ser semiespecífico

para su detección mediante la técnica de PCR cuando fueron usados por separado amplificando cinco cepas de *P. agglomerans* con el conjunto de cebadores 125.2 y una cepa de la misma especie con el conjunto de cebadores 125.3 de un total de 267 cepas analizadas (257 cepas de la especie *P. agglomerans* y 10 cepas de otros géneros). Pero, a diferencia del resto de bacterias analizadas, la cepa EPS125 fue la única que mostró señal de amplificación con ambos conjuntos de cebadores. Este resultado sugiere, el posible uso combinado de los dos marcadores moleculares para estudios de trazabilidad de la cepa EPS125 en una reacción mutiplex PCR.

P. agglomerans EPS125 presenta las características adecuadas para un potencial biopesticida comercial como la ausencia de actividad nucleadora de hielo, así como también inocuidad en plantas. Además de los ensayos en planta, esta cepa también mostró ausencia de toxicidad en mamíferos como es deseable.

La efectividad del agente de biocontrol *P. agglomerans* EPS125 para inhibir *P. expansum* fue determinada mediante ensayos de dosis-respuesta, a partir de los que se obtuvieron parámetros de eficiencia. Concretamente, siguiendo el modelo de saturación hiperbólica la cepa EPS125 fue altamente efectiva contra *P. expansum* en manzana mostrando una dosis efectiva mediana de $2.7x10^5$ a $7x10^5$ ufc/ml, y una ratio de 25-101 células de EPS125 para inactivar una espora.

El estudio del mecanismo de biocontrol empleado por *P. agglomerans* en el control de *P. expansum* se llevó a cabo mediante aproximaciones fenotípicas y estudios genéticos basados en la utilización de la técnica de mutagénesis con transposones. De acuerdo con los resultados obtenidos en la caracterización fenotípica, la producción de metabolitos antifúngicos así como también la competencia por nutrientes no parecen ser los mecanismos principales responsables de la capacidad inhibitoria de la cepa EPS125. Por el contrario, se requirió una interacción directa entre células del agente de biocontrol y esporas del patógeno es requerida para lograr la inhibición de la germinación de las esporas de *P. expansum* y su consecuente infección.

Observaciones realizadas sobre cultivos de *P. agglomerans* EPS125 crecidos en jugo de manzana muestran además de una gran cantidad de filamentos semejantes a pili de tipo IV interconectando células, agrupaciones multicelulares o 'symplasmata' envueltas por una capa de alginato. Estas observaciones parecen indicar que tanto los filamentos, los cuáles podrían ser responsables del movimiento tipo 'twitching' desembocando en la formación de microcolonias, como el alginato, la producción del cuál está influida por las condiciones de crecimiento, podrían intervenir en los primeras estadios de formación del tapete microbiano o biofilm por parte de *P. agglomerans* EPS125, así como también jugar un papel clave en la inhibición de la germinación de las esporas del patógeno.

Para demostrar realmente qué mecanismo es responsable de la actividad biocontroladora de *P. agglomerans* EPS125 se recurrió al análisis genético de mutantes defectivos en biocontrol de *P. expansum* en manzana obtenidos mediante mutagénesis aleatoria con el minitransposón GUS (mTn5SS*gusA40*). De este modo, 7 mutantes completamente defectivos en biocontrol de la podredumbre azul (m40, m439, m622, m1210, m2002, m2126 y m4015) fueron seleccionados de un total de 4032 mutantes no auxotróficos. Dichos mutantes mostraron la misma morfología colonial sobre medio mínimo ABM; curvas de crecimiento en medio LB líquido; capacidad de multiplicación en manzana a 13 y 23 ºC; señales moleculares de *quorum sensing*; y producción cualitativa de alginato. Diferencias fenotípicas respecto la cepa silvestre únicamente fueron observadas en los mutantes m2126 y m4015 en relación con la producción de filamentos mediante análisis con microscopio electrónico de rastreo.

Respecto al análisis a nivel de secuencia de ADN, éste sólo se logró en los mutantes m40, m2002, m2126 y m4015. Se obtuvo la secuencia nucleotídica flanqueante al minitransposón del mutante m40 y la proteína hipotética deducida *in silico* mostró un 35 % de identidad con secuencias de aminoácidos de la proteína dihidrodipicolinato sintasa (DHDPS) de diferentes bacterias. DHDPS es una enzima clave en la ruta de biosíntesis del *meso*-diaminopimelato (*meso*-DAP) en bacterias y plantas, el cuál es un constituyente indispensable de la pared de peptidoglicano bacteriana y precursor directo de la lisina. Debido que el mutante m40 no es auxótrofo para la lisina y posee la ruta biosintética del DAP inalterada siendo capaz de crecer en

medio mínimo; posee tres transposones insertados en su genoma; y la secuencia nucleotídica flanqueante al transposon no mostró similitud con ninguna secuencia actualmente depositada en la base de datos del GenBank, descartan a este mutante como candidato a tener en cuenta en el presente trabajo.

Las proteínas hipotéticas deducidas a partir de la secuencia nucleotídica del mutante m2126 de 876 hasta 1392 b y de 48 hasta 753 b compartió similitud con una proteína hipotética de función desconocida de *Comamonas* sp. (33 %) y con proteínas de la familia de la luciferasa bacteriana (55 %), respectivamente. De acuerdo con la bibliografía y los resultados descritos en el presente trabajo, todo parece indicar que la mutación producida en el mutante m2126 podría haber alterado una luciferasa FMN-dependiente (LuxAB) relacionada con el sistema de regulación génica vía *quorum sensing* afectando posiblemente la producción de los filamentos semejantes a los pili de tipo IV y la consiguiente formación de biofilm.

La secuencia proteica deducida a partir del mutante m4015 mostró un 41 % de identidad con lisina/ornitina N-monoxigenasas implicadas en la ruta biosintética de sideróforos del tipo aerobactina. Se sospecha que la inserción del transposón en el mutante m4015 ha podido alterar la capacidad de captar hierro del medio, dejando disponibles grandes cantidades de este elemento a *P. expansum*, el cuál es indispensable para iniciar el proceso de germinación de las esporas.

Finalmente, la secuencia nucleotídica de los mutantes m2002 y m4015 mostró un 90 % de identidad con secuencias de inserción IS1222, las cuáles mediante translocaciones y delecciones contribuyen en procesos de flexibilidad genética y adaptación al medio. A pesar que ambas secuencias nucleotídicas fueron idénticas, el punto de inserción del transposón en los dos mutantes fue distinto debido a que los patrones obtenidos en el southern y las características fenotípicas de los dos mutantes no coincidieron.

Los resultados obtenidos en el presente trabajo permiten crear un modelo hipotético de formación de biofilm en *P. agglomerans* EPS125, en donde está involucrada la inhibición de la germinación de las esporas del patógeno. En primer lugar, las células antagonistas se adhieren a la superficie de la herida de la manzana mediante una variedad de componentes celulares como filamentos semejantes a pili de tipo IV y alginato. Estos filamentos de tipo piloso se podrían unir a diferentes superficies incluyendo otras células bacterianas y esporas del patógeno produciendo movilidad de tipo 'twitching' mediante polimerización y retracción de los filamentos pilosos. Este movimiento acerca las células facilitando la formación de aglomerados muticelulares, y finalmente microcolonias en donde se incluirían las esporas del patógeno. Paralelamente, la adhesión inicial de las células antagonistas es seguida por la producción de polisacáridos extracelulares del tipo alginato, la cuál es inducida únicamente en ciertas condiciones. Además, las células EPS125 podrían ser capaces de captar hierro de medio a través de sideróforos disminuyendo su disponibilidad a las células patógenas y evitando su proceso de germinación. Entonces, el microambiente formado por la cepa EPS125 podría disminuir la disponibilidad de diferentes compuestos como agua, hierro u oxígeno que son indispensables para la germinación de conidias de *P. expansum*. Además, la cepa EPS125 produce moléculas HSL implicadas en la regulación vía *quorum sensing* de genes que podrían ser responsables de la diferenciación del biofilm y de su actividad biocontroladora. En concreto, podría estar directamente relacionado en la formación de filamentos pilosos importantes en el proceso de diferenciación de los tapetes microbianos, y en otros mecanismos de biocontrol desconocidos empleados por el agente de biocontrol. Resumiendo, el biofilm formado por *P. agglomerans* EPS125 proporciona un entorno apropiado para su crecimiento, su estructura física permite resistir estreses ambientales e inhibe la infección causada por *P. expansum* privando a las esporas de componentes indispensables como agua, oxígeno o hierro, los cuáles disparan el proceso de germinación.

En conclusión, los resultados expuestos en el presente trabajo amplían el conocimiento sobre el comportamiento de *P. agglomerans* en su ambiente natural y refuerzan la idoneidad de esta cepa como un interesante biopesticida para ser desarrollado comercialmente.

Resum

En els últims anys, el nostre grup de recerca ha portat a terme diferents prospeccions per cercar nous agents de biocontrol efectius en el control de malalties de postcollita. En una d'aquestes es va aillar la soca EPS125 per mostrar gran eficàcia biocontroladora del patogen fúngic *Penicillium expansum*, causant de la podridura blava i de grans pèrdues econòmiques en postcollita de fruita. Aquesta soca també va mostrar ser efectiva contra un ampli ventall de patògens fúngics de postcollita com *Botrytis cinerea, Monilinia laxa* i *Rhizopus stolonifer* en diferent tipus de fruites. Degut a la seva elevada eficàcia, es va plantejar desenvolupar aquesta soca comercialment; no obstant, per poder assolir aquest propòsit primer s'ha de caracteritzar el producte exhaustivament a nivell d'identificació, producció en massa, formulació, mecanisme de biocontrol i traçabilitat. Fins al moment, aspectes com la producció en massa i formulació de la soca EPS125 s'han pogut assolir amb èxit. A més a més, s'han realitzat diferents aproximacions sobre el mecanisme de biocontrol on la exclusió preventiva del patogen per colonització del nínxol i una interacció directa amb les espores fúngiques i els tubs germinatius semblen jugar un papel clau.

En el present treball es plantejà complementar la informació necessària per poder registrar el biopesticida EPS125 en un futur. En concret, els objectius van ser (i) identificació i caracterització de la soca EPS125 mitjançant proves fenotípiques i genotípiques; (ii) desenvolupament d'un mètode de traçabilitat específic per la soca EPS125 a partir de marcadors moleculars intrínsics; i (iii) determinació del mecanisme de biocontrol utilitzat per la soca EPS125 contra *P. expansum* a través d'aproximacions fenotípiques i estudis genotípics.

Segons els resultats obtinguts a partir de les proves morfològiques i bioquímiques, API 20E, patrons Biolog i d'àcids grassos, i seqüenciació del gen 16SrDNA, la soca EPS125 queda inclosa dins l'espècie *Pantoea agglomerans* (*Enterobacter agglomerans-Erwinia herbicola*). Aquesta soca mostrà trets característics que la diferenciaven d'altres espècies i de soques de la mateixa espècie. En concret, la soca EPS125 era incapaç d'utilitzar com font de carboni tween 40, però pel contrari va ser la única de metabolitzar mono-metil succinat. A més a més, en relació amb el perfil d'àcids grassos cel·lulars, aquest també va ser únic mostrant els àcids grassos 14:1 w5c, SIF1 i 15:0 ISO 3OH. En relació amb la caracterització genotípica, la soca EPS125 mostrà un polimorfisme en la longitud dels fragments de macrorestricció genòmica (MRFLP) característic format per 13 fragments (302, 270, 259, 253, 197, 179, 165, 160, 140, 110, 81 i 71 Kb) a partir de la digestió del DNA amb *XbaI*.

Dos marcadors moleculars (125.2 i 125.3) específics per la soca EPS125 van ser desenvolupats en el present treball. Cada marcador molecular mostrà ser semiespecífic per la seva detecció mitjançant la tècnica PCR quan eren utilitzats per separat amplificant cinc soques de *P.*

agglomerans amb el conjunt d'encebadors 125.2 i una soca de la mateixa espècie amb el parell d'encebadors 125.3 d'un total de 267 soques (257 soques de l'espècie *P. agglomerans* i 10 soques d'altres gèneres). Però a diferència de la resta de bacteris analitzats, la soca EPS125 va ser la única que mostrà senyal d'amplificació amb ambdòs conjunts d'encebadors. Aquest resultat suggereix la possibilitat d'utilitzar d'una manera combinada els dos marcadors moleculars per portar a terme estudis de traçabilitat de la soca EPS125 en una reacció PCR multiplex.

P. agglomerans EPS125 presenta les característiques adequades per a un potencial biopesticida comercial com és l'absència d'activitat nucleadora de gel i ser inòqua en plantes. A més a més d'aquestes característiques, aquesta soca també mostra absència de toxigeneicitat en mamífers.

L'efectivitat de l'agent de biocontrol *P. agglomerans* EPS125 per inhibir *P. expansum* va ser determinada mitjançant assaigs de dosi-resposta, a partir dels quals es van obtenir paràmetres d'eficiència. En concret, d'acord amb el model de saturació hiperbòlica, la soca EPS125 va ser molt efectiva contra *P. expansum* en poma mostrant una dosi efectiva mitjana de $2.7x10^5$ a $7x10^5$ ufc/ml, i una ratio de 25-101 cèl·lules de la soca EPS125 per inactivar una espora.

L'estudi del mecanisme de biocontrol utilitzat per *P. agglomerans* en el control de *P. expansum* es va portar a terme a través d'aproximacions fenotípiques i estudis genotípics basats en la utilització de la tècnica de mutagènesi amb transposons. Segons els resultats obtinguts en la caracterització fenotípica, la producció de metabolits antifúngics i la competència per nutrients no semblen ser els mecanismes principals responsables de la capacitat inhibitòria de la soca EPS125. Però pel contrari, era indispensable una interacció directa entre les cèl·lules de l'agent de biocontrol i espores del patogen per inhibir el procés de germinació de les espores de *P. expansum* i la seva conseqüent infecció.

Observacions realitzades sobre cultius de *P. agglomerans* EPS125 crescuts en suc de poma mostren per una banda una gran quantitat de filaments semblants als pilis de tipus IV interconnectant cèl·lules, i per l'altra banda, agrupacions multicel·lulars o 'symplasmata' embolicades per una capa d'alginat. Aquestes observacions semblen indicar que tant els filaments, els quals podrien ser responsables del moviment 'twitching' facilitant la formació de les microcolònies, com l'alginat, la producció del qual es troba influïda per les condicions de creixement (suc de poma), podrien intervenir en els primers estadis de formació de biofilm per part de *P. agglomerans* EPS125, així como també jugar un paper clau en la inhibició de la germinació de les espores del patogen.

Per demostrar realment quin és el mecanisme responsable de l'activitat biocontroladora de *P. agglomerans* EPS125 es portà a terme l'anàlisi genètic de mutants defectius en biocontrol de *P. expansum* en poma obtinguts per mutagènesi aleatòria amb el minitransposó GUS (mTn5SS*gusA40*). D'aquesta manera, set mutants totalment defectius en biocontrol van ser seleccionats a partir d'un total de 4032 mutants no auxotròfics. Aquests mutants mostraren la mateixa morfologia colonial sobre medi mínim ABM; curves de creixement en medi LB líquid; capacitat de creixement sobre poma a 13 i 23 ºC; senyals moleculars de *quorum sensing*; i producció cualitativa d'alginat. Diferències fenotípiques respecte la soca salvatge únicament van ser trobades en els mutants m2126 i m4015 en relació amb la producció de filaments.

L'anàlisi genètic a nivell de seqüència d'ADN només es va aconseguir portar a terme amb els mutants m40, m2002, m2126 i m4015. La seqüència nucleotídica flanquejant al minitransposó del mutant m40 es va obtenir i la proteïna hipotètica deduïda *in silico* mostrà un 35% d'identitat amb seqüències aminoacídiques de dihidrodipicolinat sintases (DHDPS) procedents de diferents bacteris. DHDPS és un enzim clau en la ruta biosintètica del *meso*-diaminopimelat (*meso*-DAP) en bacteris i plantes, el qual és un constituient essencial de la paret de peptidoglicà bacteriana, com també un precursor directe de la lisina. Degut a que el mutant m40 no és auxòtrof per la lisina i posseeix la ruta biosintètica del DAP inalterada essent capaç de créixer en medi mínim; conté tres transposons inserits en el seu genoma; i la seqüència nucleotídica flanquejant al transposó no mostrà similitud amb cap seqüència actualment dipositada en la base de dades del GenBank, descarten a aquest mutant como candidat a tenir en compte en el present treball.

Les proteïnes hipotètiques deduïdes a partir de la seqüència nucleotídica del mutant m2126 a partir de 876 fins 1392 b i de 48 fins 753 b compartiren similitud amb una proteïna hipotètica sense funció atribuïda de *Comamonas* sp. (33 %) i amb proteïnes de la família de la luciferasa bacteriana (55 %), respectivament. Segons la bibliografia i els resultats descrits en el present treball, tot sembla indicar que la mutació produïda en el mutant m2126 podria haver alterat una luciferasa FMN-dependent (LuxAB) relacionada amb el sistema de regulació gènica via *quorum sensing* afectant possiblement la producció de filaments semblants al pilis de tipus IV i la formació de biofilm.

La seqüència proteica deduïda a partir del mutant m4015 mostrà un 41 % d'identitat amb lisina/ornitina N-monoxigenases implicades en la ruta biosintètica de sideròfors de tipus aerobactina. Es sospita que la inserció del transposó en el mutant m4015 hagi pogut alterar la capacitat de captar ferro del medi, deixant disponibles grans quantitats d'aquest element a *P. expansum*, el qual és indispensable pel patogen per poder iniciar el procès de germinació de les espores.

Finalment, la seqüència nucleotídica dels mutants m2002 i m4015 mostraren un 90 % d'identitat amb seqüències d'inserció IS1222, les quals afavoreixen processos de flexibilitat genètica i d'adaptació al medi mitjançant translocacions i deleccions. Malgrat que ambdues seqüències nucleotídiques foren idèntiques, el punt d'inserció del transposó en els dos mutants era diferent degut a què els patrons obtinguts en el southern i les característiques fenotípiques dels dos mutants no coincidiren.

A partir dels resultats obtinguts en el present treball es pot crear un model hipotètic de formació de biofilm en *P. agglomerans* EPS125, on podria estar involucrada la inhibició de la germinació de les espores del patogen. En primer lloc, les cèl·lules antagonistes s'adhereixen a la superfície de la ferida de la poma mitjançant una gran varietat de components cel·lulars com filaments semblants als pilis de tipus IV i alginat. Aquests filaments es podrien unir a diferents superfícies com altres cèl·lules bacterianes i espores del patogen produint el moviment de tipus 'twitching' mitjançant la polimerització i retracció dels filaments pilosos. Aquest moviment aproparia les cèl·lules afavorint la formació d'aglomerats multicel·lulars i finalment, microcolònies on podrien quedar incloses les espores del patogen. Paral·lelament, l'adhesió inicial de les cèl·lules antagonistes podria anar seguida per la producció de polisacàrids extracel·lulars de tipus alginat. A més a més, les cèl·lules EPS125 podrien ser capaces de captar eficientment ferro extern via sideròfors disminuint la seva disponibilitat a les cèl·lules del patogen i evitant el seu procès de germinació. Llavors, el microambient creat per la soca EPS125 podria disminuir la disponibilitat de diferents compostos com aigua, ferro o oxigen, els cuals són essencials per iniciar el procés de germinació per part de les espores de *P. expansum*. A més a més, la soca EPS125 produeix molècules HSL implicades en la regulació via *quorum sensing* de gens que podrien ser reponsables de la diferenciació del biofilm, com també de la seva activitat biocontroladora. En concret, podria estar directament relacionat amb la formació dels filaments pilosos importants en el procès de diferenciació del biofilm i en altres mecanismes de biocontrol desconeguts i utilitzats per la cosa EPS125. En resum, el biofilm format per *P. agglomerans* EPS125 proporciona un entorn apropiat pel seu creixement, una estructura física que li permet resistir diferents tipus d'estrès ambiental, així com també podria inhibir la infecció causada per *P. expansum* privant a les espores de components indispensables per la germinació com són l'aigua, l'oxigen o el ferro.

En conclusió, els resultats exposats en el present treball amplien la informació sobre el comportament de *P. agglomerans* en el seu ambient natural i reforcen la idoneïtat d'aquesta soca com un interessant biopesticida per ser desenvolupat comercialment.

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ABBREVIATIONS

ABM	AB minimal medium
AFLP	Amplified fragment length polymorphism
AFT	Physiological buffered water
agc	Area under the growth curve
AHLs	*N*-Acyl homoserine lactones
Amp	Ampicillin
ANCOVA	Analysis of covariance
API	Analytical profile index
ASE	Asymptotic standard errors
BCA	Biological control agent
BUG™	Biolog universal growth agar
Cb	Carbenicillin
CHEF	Contour-clamped homogeneous electric field
DHDPS	Dihydrodipicolinate synthase protein
DKPs	Diketopiperazines
DMSO	Dimethyl sulfoxide
Ea	*Erwinia amylovora*
Eh	*Erwinia herbicola*
ED_{50}	Median effective dose
ELISA	Enzyme linke immunosorbent assay
EPS	Exopolysaccharide
ERIC	Enterobacterial repetitive intergenic consensus
FAME	Fatty acid methyl ester
FT-IR	Fourier transform-infrared spectroscopy
GB	Glycine betaine
GFP	Green fluorescent protein
GLM	General linear model
GMO	Genetically modified organism
gt	Generation time
GUS	β-glucuronidase
GY	Glucose yeast extract
H	HEPES
HG	HEPES with 10 % glycerol
HR	Hypersensitive response
HS	Hyperbolic saturation model
IAA	Indol acetic acid
INA	Ice nucleation activity
IR	Infrared
IS	Insertion sequence
ISR	Induced systemic resistance
IVET	In vivo expression technology
ivi	In vivo-induced
Km	Kanamycin
LB	Luria bertrani
LuxAB	Bacterial FMN-dependent luciferase
Mer	F420-dependent N5, N10-methylenetetrahydromethanopterin reductases
meso-DAP	*meso*-diaminopimelate
MRFLP	Macrorestriction fragment length polymorphism

MSE	Mean square error
NA	Nutritional agar medium
NADPH	L-lysine 6-monooxygenase
NASBA	Nucleic acid sequence based amplification
NMP	Most probable number
NOI	Niche overlapping index
OMPs	Outer-membrane proteins
orf	Open reading frame
Pa	*Pantoea agglomerans*
pBS	pBluescript plasmid
PCN	Phenazine-1-carboxamide
PCR	Polymerase chain reaction
PDA	Potato-dextrose agar
Pf	*Pseudomonas fluorescens*
PFGE	Pulsed field gel electrophoresis
PLS	Pili-like structures
purA	Purine auxotrophs
Q-PCR	Quantitative-PCR
QC-PCR	Quantitative competitive-PCR
RAPD	Random amplified polymorphic DNA
REA	Restriction endonuclease analysis
REP-PCR	Repetitive extragenic palindromic-PCR
RFLP	Restriction fragment length polymorphism
Rif	Rifampicin
RT-PCR	Reverse transcription-PCR
SAR	Systemic acquired resistance
SCAR	Sequence characterized amplified region
SDM	Semidefined medium
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electronic microscope
SIF	Sum in feature
Sm	Streptomycin
Spc	Spectinomycin
SsuD	Alkanesulfonate monoxygenases
STM	Signature-tagged mutagenesis
TAE	Tris-acetate-EDTA
Tc	Tetracycline
TEM	Transmission electronic microscope
TSBA	Tryptone soya B agar
unk	Unknown
VBNC	Viable but not cultivable
VCP	Viable count by plating

Introduction

1. Control methods of plant disease during production and postharvest

During the processing of agricultural products, significant economic losses occur due to the action of pathogenic microorganisms. Physical and biochemical strategies used by plants to defend from pathogens such as accumulation of phytoalexines, modification of cellular wall and synthesis of hydroxylases, chitinases and β-1,3-glucanases (Mari and Guizzardi, 1998), are not sufficient to avoid the infection process. Up to now, to decrease these losses conventional treatment based on the application of bactericides and fungicides have been used, but in an unfair way. This system has been the most used during years because has showed to be effective, persistent on time, with an easy application and prompt results. However, as a consequence of this performance, chemical residues accumulated on agricultural produce affecting directly to consumer and environment and developing resistance of some pathogens to the treatment.

Currently, there is a trend to rational use of pesticides, as well as, to reduce the number of authorized active substances to those unavoidable, more selective, less toxic and with lower negative environmental impact (Ragsdale and Sisler, 1994; Gullino and Kuijpers, 1994; European Comission, 2003). European Union has established directive 91/414/EEC which regulates the register of pesticides. In the case of postharvest, the few permitted chemical products nowadays used to control postharvest fruit diseases in Spain are captan, imazalil, iprodione, tiofanat metil, ortophenylphenol and thiabendazole (MAPA, 2005). Therefore, there is an urgent need for alternative or complementary methods to avoid or decrease the impact of plant disease on production and postharvest.

It is well known that fruit and vegetables are susceptible products to spoiling. Wounds present on produce during storage, often as a result of harvesting and transportation, give an easy access to pathogens (Spadaro and Gullino, 2004) and the high water content in fruit allows the pathogen attack (Harvey, 1978). Among the most important postharvest pathogens of fruit, the fungal pathogens *Penicillium* spp, *Botrytis cinerea*, *Monilinia laxa* and *Rhizopus stolonifer* have been broadly documented (Ogawa, 1995; Pla *et al*., 2005).

Actually, the technology most frequently used to control postharvest diseases is based on storage methods of fruit and vegetables under refrigeration combined or not with modified atmosphere. In this way, transpiration of produces is decreased, biochemical changes of senescence of fruit are retarding, and spore germination and growth of fungus are inhibited. In parallel, other technologies widely used are the application of heat treatments as hot water, vapour heat and hot air (Lurie, 1998), or the addition of plant extracts and synthetic chemical fungicides

to fruit and vegetables before their storage in cool storage chambers (Eckert and Ogawa, 1988; Bautista-Baños *et al.*, 2000). Therefore, the best resort to avoid postharvest diseases is applying careful practices during harvest and store processes, minimizing production of wounds on fruit.

Due to the consumer demand, during the past years have increased efforts to develop alternative or complementary methods to pesticide such as biological control (El-Ghaouth *et al.*, 1995; Janisiewicz *et al.*, 1995). Biological control consists of natural occurring microorganisms known as Biological Control Agents (BCAs) with the capacity to avoid or decrease the development of pathogen and consequently its infection. Therefore, biological control fits in well with the concept of sustainable agriculture because it exploits natural cycles with reduced environmental impact (Spadaro and Gullino, 2004).

2. Biological control

The National Academy of Sciences of United States of America defines biological control as "the use of natural or modified organisms, genes or gene products, to reduce effects of undesirable organisms and to favour desirable organisms such as crops, trees, animals, insects and beneficial microorganisms" (NAS 1987, cited by Thomashow and Weller (1996)).

As it has been commented above, most postharvest infection of fruit and vegetables occur through wounds. So it is necessary the application of effective BCAs able to colonize wounds inhibiting the development of pathogen. However, a complex interaction between antagonist, pathogen, host resistance, wound response, and other interacting microorganisms occurs involving a possible variation of biocontrol levels. In case of biological control of postharvest diseases, frigoconservation chambers show stable storage environment with constant temperature, composition gas and humidity, parameters that can be controlled to switch the host-pathogen-antagonist equilibrium towards the antagonist (Wilson *et al.*, 1987; Pusey *et al.*, 1988). In addition, the capacity to control physical parameters allows that laboratory results can be made to more nearly approximate postharvest conditions. Moreover, postharvest biocontrol is especially feasible because harvested fruit are accessible to treatment with antagonists and the same facilities used for apply fungicides can be used to treat produce with BCAs.

In general, it is wanted to find an ideal antagonist with the following characteristics: (i) genetic stability; (ii) efficacy at low concentrations and against a wide range of pathogens on a variety of fruit and vegetables; (iii) simple nutritional requirements; (iv) survival in adverse environmental conditions; (v) growth on cheap substratum in fermenters; (vi) lack of pathogenicity for the host plant and no production of metabolites potentially toxic to humans; (vii) resistance to the most frequently used pesticides; and (viii) compatibility with other chemical and physical treatments (Wilson and Wisniewski, 1992).

2.1. *Pantoea agglomerans* as effective biological control agent

During the past 20 years, several microorganisms have been the focus of considerable research for the use as BCAs against different postharvest fungal pathogens like *Aspergillus* spp., *Botrytis* spp., *Monilinia* spp., *Penicillium* spp. and *Rhizopus* spp. (Lima *et al.*, 1997; Viñas *et al.*, 1998; Zahavi *et al.*, 2000; Northover and Zhou, 2002; Droby *et al.*, 2002; Bonaterra *et al.*, 2003; Francés *et al.*, 2006) and in several fruit types as peach, nectarine, apple, pear, citrus, cherry, kiwi, grape, apricot, plum and strawberry (Pusey and Wilson, 1984; Janisiewicz and Roitman, 1987; Wilson *et al.*, 1987; Mclaughlin *et al.*, 1992; Madrigal *et al.*, 1994; Chand-Goyal and Spotts, 1997; Cook *et al.*, 1999; Nunes *et al.*, 2001; Bonaterra *et al.*, 2003; Francés *et al.*, 2006). So far, the genera *Pseudomonas*, *Erwinia* and *Bacillus* have been described as the most important BCAs into the bacterial group. Concretely, as Table 1 shows different strains of *Erwinia herbicola* (synonymous of *Pantoea*

agglomerans and *Enterobacter agglomerans*), that have been reported being efficient biocontrol agents against important bacterial and fungal pathogens of aerial and root part of plants (Vanneste *et al.*, 1992; Yuen *et al.*, 1994; Montesinos *et al.*, 1996; Zhang and Birch, 1997; Stockwell *et al.*, 1998) and in fruit in postharvest (Bryk *et al.*, 1998; Teixidó *et al.*, 2001; Nunes *et al.*, 2001; Bonaterra *et al.*, 2003; Francés *et al.*, 2006).

Table 1. Examples of *P. agglomerans* as BCAs of important bacterial and fungal pathogens on different hosts.

Pathogen	Host	Reference
Botrytis cinerea	Strawberry fruit	Francés *et al.*, 2006
Erwinia amylovora	Apple blossoms	Vanneste *et al.*, 1992
Monilinia laxa and *Rhizopus stolonifer*	Apricot, cherry, nectarine, peach and plum fruit	Bonaterra *et al.*, 2003; Francés *et al.*, 2006
Penicillium expansum	Apple and pear fruit	Francés *et al.*, 2006
Pseudomonas syringae pv *syringae*	Barley	Braun-Kiewnick *et al.*, 2000
Puccinia recondita, Fusarium culmorum	Wheat	Kempf *et al.*, 1993
Pythium sp.	Cotton	Nelson, 1988
Rhizopus stolonifer and *Botrytis cinerea*	Apple	Teixidó *et al.*, 2001; Nunes *et al.*, 2001
Sclerotinia sclerotorium	Bean	Yuen *et al.*, 1994
Uromyces appendiculatus	Bean	Yuen *et al.*, 2001
Xanthomonas campestris pv *armoriaceae*	Radish	Han *et al.*, 2000

Ewing and Fife included clinical strains identified as *Enterobacter agglomerans* in the species *E. herbicola* (Brenner *et al.*, 1984). Later, *E. herbicola* was defined as *P. agglomerans*, a new species, including all bacteria with the same characteristics of *E. herbicola* and *E. agglomerans* associated to plants (Gavini *et al.*, 1989). The taxonomic structure of this group has been studied by means of phenotypic (Ewing and Fife, 1972; Dickey, 1979; Thomson *et al.*, 1981; Mergaert *et al.*, 1984) and genotypic characteristics (Brenner *et al.*, 1984; Kwon *et al.*, 1997). It is generally accepted the consideration of the synonyms *P. agglomerans*, *E. herbicola* and *E. agglomerans* species. However, in the present work, we have chosen the term *P. agglomerans*.

Biological control agents generally provide a crop protection with a low environment risk associated. However, this does not mean that they did not have hazards to health and the environment. For this reason, several attempts have been performed to classify human and animal pathogens according to the risks that they would present to the human and animal community and to their field use whether they escaped from the laboratory. The European Parliament and the Council have published the Directive 2000/54/EC on the protection of workers from risks related to exposure to biological agents at work. This Directive refers to biological agents may be able to provoke any infection, allergy or toxicity. But in fact, the classification of infectious biological agents performed by the European Community is based on the level of risk of infection.

If we flick through the Belgian biosafety server list of bacteria presenting a biological risk for healthy plants, we find *P. agglomerans* (*E. herbicola*, *Erwinia milletiae*) into the biological risk group 2. Group 2 comprises biological agents that can cause human disease and might be a hazard to workers; it is unlikely to spread to the community and there is usually effective prophylaxis or treatment available. In case of the classification performed by the European Community and United Kingdom, it only appears the genus *Enterobacter* with a biological risk of class two. Instead, United States classification includes neither genus Enterobacter, nor species of *P. agglomerans* as microorganisms with a biological risk associated. Hence, some disagreements exist between, and even within individual states to allocate biological agents to one risk group. This is due to the variability of agent characteristics, work conditions, host models and other factors. For these reason, classification tables should be used only as a guide for the comparison of the relative hazard levels of the several agents. Moreover, it should be considered that species belonging to the *Erwinia* genus present a high heterogeneity (Kwon *et al.*, 1997). In fact, it has been reported some strains able to protect fruit and vegetable against invasion of the pathogen (Beer *et al.*, 1984;

Johnson *et al.*, 1993) and even strains like EPS125 that were negative in acute toxicity tests in mammals (Montesinos *et al.*, 2001). For this reason, it is not reasonable to consider all members of the species *P. agglomerans* at risk level two, as the Belgium system of classification of pathogens shows. Finally, it has been extensively reported that *P. agglomerans* is a component of the dominant microbiota of many plants (Elvira-Recuenco and van Vuurde, 2000; Lindow and Brandl, 2003) and it appears clear that we eat reasonable amounts of this bacteria upon consumption of fresh fruit.

P. agglomerans is a gram negative eubacterium with peritrichous flagella, facultative anaerobic and with the capacity to produce yellow pigments on different culture media (Brenner *et al.*, 1984). It is a normal inhabitant in many environments and is found frequently associated to the ground (Yeung *et al.*, 1998); water (Mosso *et al.*, 1994); as epiphyte on plants (Elvira-Recuenco and van Vuurde, 2000; Lindow and Brandl, 2003); and occasionally on humans (de Champs *et al.*, 2000).

Many strains of *P. agglomerans* have several aptitudes as biocontrol agents of plant diseases due to his ubiquity and the capacity to produce antimicrobials metabolites. This last property could supply good aptitudes to colonize plant surfaces (Cook and Baker, 1983) and compete efficiently with indigenous microbial communities in several environments (Leisinger and Margraff, 1979; Ishimaru *et al.*, 1988; Défago and Haas, 1990; Chenu, 1993). Different studies have been performed in relation with the capacity of *P. agglomerans* to synthesize herbicolines, a type of peptide antibiotic that acts against fungus containing sterols (Greiner and Winkelmann, 1991; Tenning *et al.*, 1993), and pantocines, which inhibit the synthesis of specific aminoacids in *E. amylovora* (Sutton and Clardy, 2001; Wright *et al.*, 2001).

In a screening program performed in our laboratory with the aim to isolate naturally occurring bacteria from plants with potential application for biocontrol (Montesinos *et al.*, 1996), a strain of *P. agglomerans* named EPS125 was selected. Concretely, it was isolated from the surface of a Doyenne du Comice pear being highly effective in control *P. expansum* in apple and pear fruit (Vázquez, 1998; Francés, 2000). For developing and establishment of a BCA as a biopesticide, in this case EPS125, an overall of steps are required to finally arrive to his commercialization (Montesinos, 2003a).

Several assays have been performed with the aim to determine the biocontrol spectrum of strain EPS125. As shows Table 2, EPS125 is an excellent candidate for biopesticide having the capacity to control a wide range of important postharvest pathogens in comparison to other bacteria used as BCA of fruit rot that are more specific (Janisiewicz and Roitman, 1988; Francés, 2000; Bonaterra *et al.*, 2003; Francés *et al.*, 2006) or control several postharvest pathogens in a host limited range (Utkhede and Sholberg, 1986; Janisiewicz and Marchi, 1992; Teixidó *et al.*, 2001; Nunes *et al.*, 2001). For example, it inhibits blue mould in pear and apple fruit, either in commercial cold storage conditions under controlled atmosphere or in simulated market conditions, showing efficacy results that not significantly differ from treatments with chemical fungicides (Francés, 2000).

Table 2. Antagonism spectrum of EPS125 on different fruit

Pathogen	**Disease**	**Fruit**	**Reference**
Botrytis cinerea	Grey mould	Strawberry	Francés *et al.*, 2006
Monilinia laxa	Brown rot	Apricot, peach, nectarine, plum and cherry	Bonaterra *et al.*, 2003; Francés *et al.*, 2006
Rhizopus stolonifer	Black mould	Apricot, peach, nectarine, plum and cherry	Bonaterra *et al.*, 2003; Francés *et al.*, 2006
Penicillium expansum	Blue mould	Apple and pear	Francés, 2000; Bonaterra *et al.*, 2003

Moreover, EPS125 is also able to control grey mould of strawberry and brown and soft rot of stone fruit under storage conditions varying from controlled atmosphere cold storage to room temperature. In addition, the strain EPS125 has been object of toxicological studies

showing that it produces neither primary dermal nor eye irritation on rabbit, and its acute oral toxicity LD_{50} on rats is higher than 10^{11} cfu/kg, as it is desirable (Montesinos *et al.*, 2001).

2.2. Development of microbial pesticides for biological control

The development of a microbial pesticide requires many different studies, but usually the first stages include the following steps: (i) select possible antagonists by means of a screening method able to analyze a high number of isolates, (ii) develop a mass production method, (iii) find the appropriate formulation that allows to increase biocontrol activity and ensure its stability, (iv) determine mechanisms of biocontrol to improve its activity and (v) develop a monitoring system to detect and quantify the antagonist in the environment. Finally, this overall information will be documented in a more extensive dossier including additional studies such as toxicology or environmental impact with the aim to register the biopesticide (Figure 1).

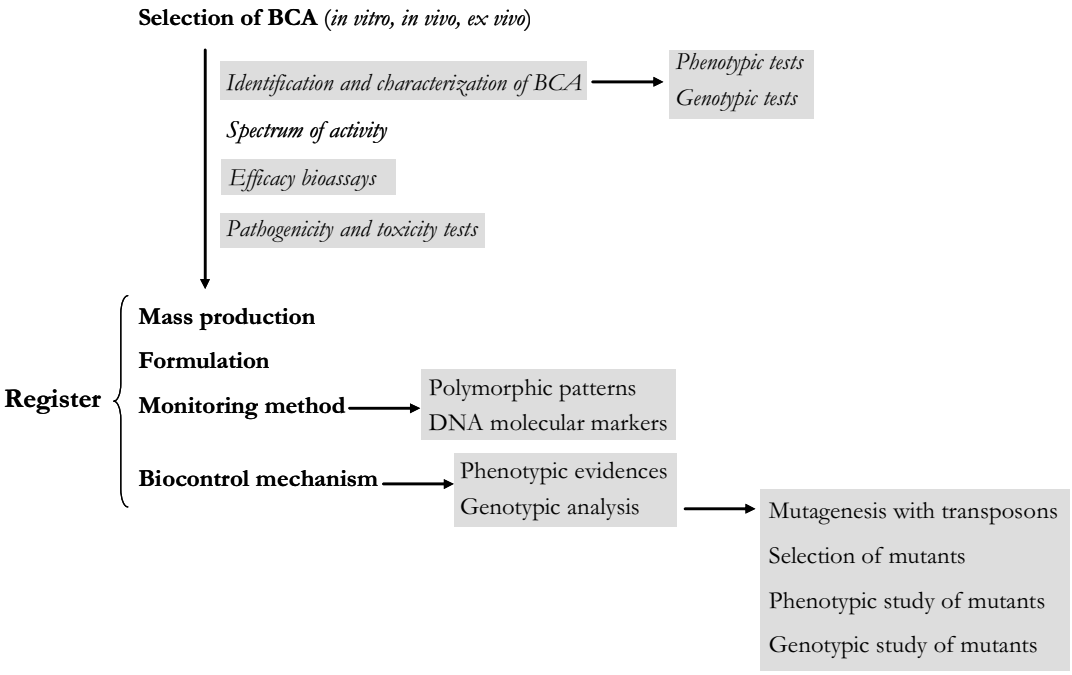

Figure 1. Scheme of overall steps required for development of a biopesticide and partially followed in the present work (grey squares).

Selection

The first stage consists of the isolation of microorganisms able to interfere with the biological cycle of plant pathogens or pests. Thus, it is strongly critical to decide where will be taken samples to isolate BCAs, because depending on the screening method used it will be selected a type or another of microorganism. Usually, they are taken at places or in certain materials where have had evidences of their presence such as dead arthropods, disease-suppressive soils or healthy plants in epidemic areas (Montesinos, 2003a). Another interesting place described by some authors to find candidates for BCAs is near of the infection place of the pathogen (Handelsman *et al.*, 1990). Finally, microorganisms with biocontrol capacity could be isolated from the same natural

environment where will be introduced afterwards. Likewise, the surface and wounds of fruit are places with high probability to find effective postharvest BCAs.

The second stage of biopesticide development is to choose a screening method, which has to be simple and reproduce natural conditions that will occur in plant and postharvest environment. At the moment, screening methods are based on efficacy bioassays that can be performed *in vitro*, *ex vivo* and *in vivo*, and finally pilot trials under real conditions of application (field, greenhouse and postharvest) (Montesinos *et al.*, 1996). The ideal method to isolate antagonists consists on direct inhibition of disease *in plant*, nonetheless it is material and time consuming, becoming an impractical method of screening (Sivan and Chet, 1992). From among screening systems described in the bibliography, *in vitro* assays are the most used because are relatively easy to perform and prompt results are obtained. However, results of the selected BCA sometimes do not fit well with control values obtained using plant material. It might be due to the limited capacity of the antagonist to survive on the new environment (Mercier and Wilson, 1994). Therefore to select a BCA able to survive in intact plant surfaces, a variation from *in vitro* screening has been developed. Once antagonists have been isolated from *in vitro* screening, they are analyzed by means of inhibition bioassays on plant material, verifying that maintain its biocontrol capacity. In this way, there is a risk that some effective biocontrol strains on plant or fruit would not be chosen in the *in vitro* screening (Andrews, 1985). For example, BCAs that act by means of competition for space would not be selected (Andrews, 1992). The ideal screening method has to simulate to more nearly approximate natural conditions but using *in vitro* assays. In this context, it has been described an *ex vivo* screening which look for BCAs with capacity to control pathogen directly on the host tissue (immature fruit, leaves, flowers or whole plants) under controlled environment (Nelson and Craft, 1992; Gould *et al.*, 1996). Finally, it has been developed a rapid and simple screening method named selective enrichment based on the use of an extract, a suspension of microorganisms associated to the plant or fruit. The extract is coinoculated with the pathogen in host tissue and the tissues that showed inhibition activity against the pathogen are selected (Vázquez, 1998). Later, the several microorganisms isolated from the extract in pure culture are submitted to new inhibition bioassays on plant material, and parallel *in vitro* inhibition assays are also performed with each one (Schisler and Slininger, 1997).

When a microorganism has been selected for its capacity to control a specific pathogen, it is necessary to proceed to its identification and characterization by phenotypic analysis as for example API, Biolog or FAME; and genotypic techniques such as sequencing of 16SrDNA gene, or genomic patterns by means of MRFLP or RAPD. These studies provide the information needed for registration and patenting the biocontrol strain, as well as for recognition and quality checking during production of the biopesticide (Plimmer, 1993; Lemanceau *et al.*, 1995).

To develop a commercial biopesticide it is interesting also to perform efficacy bioassays in several pathosystems to determine the spectrum of activity of the selected BCA on different fruit. In addition, knowledge of the antagonist-pathogen inoculum density relationships can show what population levels of the antagonist are required to achieve adequate disease control (Montesinos *et al.*, 1996; Larkin and Fravel, 1999). Employing this kind of bioassays makes possible to study the effect of the pathogen and BCA concentrations on the incidence and severity of disease for each pathosystem analyzed. In this context, dose-response models have been developed to obtain quantitative parameters that allow for one hand, to compare control efficacies of antagonist; and for the other hand, to know the ratio of cells of BCA needed to inhibit one cell of the pathogen providing 50% of control disease (Johnson, 1994; Montesinos *et al.*, 1996; Smith *et al.*, 1997; Bonaterra *et al.*, 2003). Finally, it is extremely important to demonstrate that the biopesticide does not have target effects on plants, animals and environment. Rather, it is necessary an absence of plant pathogenicity, which is indicated by the absence of reaction of hypersensitivity in solanaceous plants, as well as the lack of acute toxicity on mammals (Montesinos, 2003a).

Mass production

During the selection process of a microbial pesticide, studies about the production at industrial scale have to be performed to get a stable product through time for delivery.

It is a key issue that mass production has to be rapid, efficient and inexpensive. It is fundamental to find a medium of culture that provide maximum biomass production, minimum cost and an optimum system of fermentation (continuous, discontinuous, solid or liquid), maintaining biocontrol efficacy (Spadaro and Gullino, 2004). One of the main limiting factors during development of a biopesticide is to find a treatment to increase the shelf-life of the microorganism for the storage period depending of the agent nature (Powell and Jutsum, 1993; Jones, 1993). Starting from liquid state culture of the antagonist, there are several maintenance systems described, for example refrigeration, freezing adhering cryoprotectant substances or keeping as a dehydrated product (Montesinos, 2003a). Methods based on dehydratation such as lyophilization and spray-drying allow optimum conditions of storage, handling and formulation of the microorganism. At industrial level, spray- or fluidized-drying methods are the most used because it achieves a low cost product (Montesinos, 2003a).

Nowadays, a mass production method for the biopesticide EPS125 has been developed at semi-industrial scale by liquid fermentation. The fermentation process is performed in a bioreactor of 50 l, which has a production capacity near to 1000 l of product at the recommended application dose (1-5 x 10^{7} cfu/ml) in 8 h. This product can be centrifuged, deep-freezed and dehydrated by liophylization or atomization with skimmed milk or dextran, maintaining its shelf life for several months. In this way, this product can be easily reconstituted with water and applied on fruit by spraying or immersion (Montesinos *et al.*, 2001).

Formulation

An accurate formulation can be decisive to improve efficacy and extend the product shelf-life, facilitating storage for commercially acceptable periods of time (Janisiewicz and Jeffers, 1997). The application of biocompatible additives to dehydrated or liquid product can protect and stimulate the establishment of the antagonist on the host surface, as well as increase the survival of the BCA under stress conditions of field such as nutrient limitation, ultraviolet light or low water potential (Andrews and Harris, 2000; Lindow *et al.*, 2002). The addition of glycine and trehalose to the culture medium increases the biocontrol activity of the antagonistic yeast *Candida sake* against *P. expansum* on apple (Janisiewicz *et al.*, 1992). Moreover, several assays have been performed to test synergistic effect of the BCA combined with different substances of disease control. For example, El Ghaouth *et al.* (2000) have developed a postharvest biocontrol product called "bioactive coating" consisting on a combination of yeast antagonist with glycolchitosan, a molecule with antifungal properties. Likewise, Tian *et al.* (2005) have proved an effective combination of yeasts *Cryptococcus laurentii* and *Rhodotorula glutinis* with silicon against *Alternaria alternata* and *P. expansum* of jujube fruit, respectively.

In the last years our laboratory has carried out several assays to improve survival and biocontrol capacity of strain EPS125 by means of different formulations. With this in mind, Bonaterra *et al.* (2005) proved that the osmoadaptation of *P. agglomerans* EPS125 by saline osmotic stress and Glycine Betaine (GB) increased tolerance to desiccation and its survival on surface of apple fruits. This result fits in well with reports on literature with other BCAs where the accumulation of osmoprotectants as trehalose and GB reaches a high tolerance to desiccation and other interesting beneficial effects under stress conditions (Vandijck *et al.*, 1995; Teixidó *et al.*, 1998; Welsh and Herbert, 1999; Harman *et al.*, 2006). Another strategy to increase control of postharvest pathogens is to combine the strain EPS125 with fungicides at reduced doses. This BCA has shown to be tolerant to common fungicides used in agriculture as Folper, Imazalil, Miclobutanil, Tiabendazol, Tiram, Benomilo, Captan, Procimidona, Tebuconazol, Difenilamina and Metil-tiofanato (Montesinos *et al.*, 2001).

In any way, to develop an effective formulation that improves the antagonistic activity of the BCA and its application systems is interesting to know the mechanism of action that confer its activity.

Monitoring

When a BCA is introduced at large scale into the environment there is a need to develop monitoring methods that include detection and quantification of the antagonist strain (Table 2). This method will permit to study the adaptation, survival and impact of the BCA on the environment, as well as to bear out if the techniques of application of the antagonist are optimum.

Table 2. Monitoring methods described on bibliography to detect and quantify specific microorganisms (Modified from (Ryder, 1995))

Monitoring method	Detection
Spontaneous mutants resistant to antibiotics	Growth on selective culture medium (VCP, MPN)
Immunological methods	Detection with specific antibodies (ELISA and IF)
Hybridization with DNA probes	Hybridization with target sequence
Introduction of genetic markers-reporters	Antibiotic resistance, GFP, luciferase, β-glucoronidase, histidine auxotrophs, new metabolic abilities, heavy metal resistance
Polymorphic patterns	RAPD, REP-PCR, ERIC-PCR, BOX-PCR, AFLP, RFLP, MRFLP, PCR-RFLP
Intrinsic molecular markers	PCR, Q-PCR, QC-PCR, Real-Time PCR, NASBA

So far, the classic monitoring method most used is based on obtaining a spontaneous mutant that has acquired resistance to an antibiotic. For instance, our laboratory has selected a spontaneous mutant of the biocontrol agent of fire blight *Pseudomonas fluorescens* 62e Nal resistant to nalidixic acid (Pujol *et al.*, 2005) and a mutant of the biocontrol agent of postharvest pathogens *Pantoea agglomerans* EPS125 Rif resistant to rifampicin (Bonaterra *et al.*, 2003).

Currently, some laboratories employ serological techniques to detect a specific microorganism, for instance Enzyme Linke Immunosorbent Assay (ELISA) which is based on the use of monoclonal or recombinant antibodies (Allen *et al.*, 2006). However, in the last years systems based on reporter gene and direct detection of nucleic acid have been used to detect plant associated microorganisms. These methods have become useful tools for evaluating *in situ* gene expression, colonization, mechanism of action and monitoring of release (Delorenzo *et al.*, 1990; Aguilar *et al.*, 2002; Montesinos, 2003b). Reporter gene systems are based on variants of the antagonistic strains with a genetically stable marker which maintain their biocontrol capability and do not show detectable differences between the wild type and the transformants. In comparison with classic microbiological methods, these kinds of markers are more stable and its expression is independent of the type of culture media used. The introduction of genetic markers to antagonists can be performed by means of plasmids. However, the use of this transformation vectors implies the possibility of horizontal transference to other microorganisms, including human and animal pathogens, as well as the loss of plasmid in the absence of selective pressure (Griffiths *et al.*, 1990). Nonetheless, to avoid the disadvantages of using plasmids, another system based on the introduction of the marker gene to the chromosome by means a suicide plasmid containing a transposon has been proposed (Mahaffee *et al.*, 1997).

Early experiments of transformation with marker genes have been successful, for instance *Metschnikowia pulcherrima* was transformed with the green fluorescent protein gene (Nigro *et al.*, 1999), *P. fluorescens* 89B-27 with a bioluminescent marker (*lux* operon) (Mahaffee *et al.*, 1997) *Candida oleophila* with the β-glucoronidase gene (Chand-Goyal *et al.*, 1998), and histidine auxotrophs of *C. oleophila* with *HIS3, HIS4 and HIS5* genes (Chand-Goyal *et al.*, 1999). Since the introduction of genetically modified (GM) crops in 1996, impact on associated microbiota and the

remote possibility of horizontal transfer of antibiotic selectable markers from transgenic plants to plant-associated bacteria, and their subsequent transfer from these bacteria to human or animal bacterial pathogens, has been a cause of concern for the large-scale commercial introduction. However, in the study performed by Badosa *et al.* (2004) any event of transference of ampicillin gene from transgenic plants to epiphyte microbiota was not detected.

Therefore, to avoid the introduction of exogenous DNA into antagonists, non invasive techniques have been developed to identify intrinsic natural genotypic markers based on polymorphic patterns, but it is a difficult and time consuming process. Most methods are related to the Polymerase Chain Reaction (PCR) such as Random Amplified Polymorphic DNA (RAPD) (Pujol *et al.*, 2005), Repetitive Extragenic Palindromic-PCR (REP-PCR), Enterobacterial Repetitive Intergenic Consensus (ERIC) (Versalovic *et al.*, 1991) and Amplified Fragment Length Polymorphism (AFLP) (Yunis *et al.*, 1991); DNA digestion with restriction enzymes for instance Restriction Fragment Length Polymorphism (RFLP)(Kiko *et al.*, 1979); or combination between them such as PCR-RFLP (Al Dahouk *et al.*, 2005). Finally, comparing polymorphism patterns of the BCA with isolates from the same species, strain specific fragments can be identified sequenced and characterized as Sequence Characterized Amplified Region (SCAR). With the information of the SCAR sequence, specific primers to detect the antagonist by PCR analysis can be designed (Paran and Michelmore, 1993; Pujol *et al.*, 2005).

Routinely, to perform the quantification of the BCA, Viable Count by Plating (VCP) or Most Probable Number (NMP) based on the spread on semiselective medium supplemented with the appropriate antibiotic have been used (Pujol *et al.*, 2005). This classic microbiological method is frequently used on several studies of postharvest biocontrol because it is available for laboratories, special equipment is not needed and is easy to achieve. Usually, antagonistic strains are selected by spontaneous mutations resistant to an antibiotic remaining stable most phenotypic and genotypic characteristics and allowing its monitoring in biocontrol assays at field (Bonaterra *et al.*, 2003; Pujol *et al.*, 2005). For instance, our laboratory has performed several postharvest assays with a spontaneous mutant of strain EPS125 resistant to 100 μg/ml of rifampicine (Bonaterra *et al.*, 2003; Pujol *et al.*, 2005). However, this method could have interference by microorganisms from the authoctonous microbiota which present resistance to the same marker. The presence of antibiotic resistance in natural populations of bacteria associated to plants has been reported. A study performed by Badosa *et al.* (2004) about the transference of gene of ampicillin from corn Bt176 to associated microbiota, a high proportion of ampicillin resistant bacteria occurring naturally in agricultural fields fertilized was found. Another disadvantage about the use of spontaneous variants of the wild type is that the mutation may exert pleiotropic effects on its fitness (Mahaffee *et al.*, 1997). Finally, it is difficult to have a selective medium for the BCA, as well as to detect cells of the antagonist when enters to a state of Viable But Not Cultivable (VBNC). This conflict could be solved by combining VCP or MPN with a PCR system. In this context, Pujol *et al.* (2005) have compared two quantification methods, VCP against MPN combined with PCR using SCAR primers to determine population levels of *P. fluorescens* EPS62e Nal, an effective BCA of fire blight, on pear plants under green house conditions. Both methods showed to be useful in monitoring of the antagonist, showing the same sensitivity level for all concentrations tested. In the last years, have been developed several techniques basing on PCR, that allow monitoring of microorganisms, for instance PCR-based dilution end point or Quantitative-PCR (Q-PCR) (Cross, 1995; Zimmermann and Mannhalter, 1996), Quantitative Competitive-PCR (QC-PCR) (Cross, 1995; Zimmermann and Mannhalter, 1996) and Real Time PCR (Higuchi *et al.*, 1992). The molecular techniques based on DNA detection are able to detect cells in state VBNC (Knight, 2000); nonetheless, they cannot distinguish between viable from not viable cells. To solve this problem, a molecular tool based on detection of mRNA has been developed NASBA (Nucleic Acid Sequence based Amplification) (Demain, 2001), as well as combine DNA fingerprinting methods with selectable antibiotic resistance.

So far, microbiological monitoring methods based on culture on media supplemented with rifampicin (100μg/ml) has been used to detect and quantify a spontaneous mutant of EPS125 Rif in several conditions (Bonaterra *et al.*, 2003; Francés *et al.*, 2006). However, population levels may be overestimated due to presence of other bacteria resistant to the same antibiotic in natural samples and antagonistic cells may be in viable but not cultivable state and would not been detected (Badosa *et al.*, 2004; Bakker *et al.*, 2006; Rattray *et al.*, 1993). Then, to avoid these disadvantages it would be interesting develop molecular monitoring methods of the parental strain EPS125.

Mechanism of action

Information about the mechanism of action for most of the antagonists is still incomplete due to difficulties in analysing the complex interactions between host, pathogen, antagonist and other microorganisms present. Knowledge of the mode of action can permit establishment of optimum conditions for the interaction between the BCA and the pathogen and the design of appropriate formulations and methods of application (Spadaro and Gullino, 2004).

Several biocontrol mechanisms have been described including antibiosis, competition for nutrients and space, induction of mechanisms of resistance in the host tissue, quorum quenching and direct interaction between the antagonist and the pathogen including parasitism. Actually, most biocontrol agents not only use one mechanism of biocontrol, but disease control is the result of a combination of several mechanisms.

Antibiosis

Some of the most active BCAs are bacteria producing antibiotics or toxins that negatively affect target organisms. For example a strain of *Bacillus subtilis* produces iturin, a powerful antifungal peptide (Gueldner *et al.*, 1988), as well as gramicidin S (Edwards and Seddon, 2001); *Pseudomonas cepacia* synthesizes pyrrolnitrin, which can control *B. cinerea* and *P. expansum* attacks on pome fruit (Janisiewicz *et al.*, 1991) and *E. herbicola* produces pantocine, antibiotic that inhibits the synthesis of aminoacids in *E. amylovora* (Wodzinski *et al.*, 1996). The main concerns related to the use of antibiotics in food products are for one hand, the residues accumulated on consumable products that can affect health of humans and livestock (Diekman and Green, 1992; Cheeke, 1995) and for the other hand, there is the possibility of development of resistant pathogens to these compounds (Spadaro and Gullino, 2004). However, this is not the case in many BCAs producing antibiotics because these antibiotics have not cross resistance with antibiotics clinically used. However, most BCAs produce more than one antibiotic, decreasing the probability to acquire resistance to multiple antibiotics, and total exposure of the pathogen population to the antibiotics is low because the populations of BCAs are localized minimizing selection pressure. Nonetheless, it has been reported cases where plant pathogens have acquired resistance to the antibiotics produced by some BCAs, such as the pathogen *Agrobacterium tumefaciens*. In this case the plasmid pAg K84 from the antagonist *Agrobacterium radiobacter*, which carried genes for synthesis and resistance to the antibiotic agrocine 84, was transferred to the pathogen (Vicedo *et al.*, 1993). Therefore, the main problem is the loss of efficacy of control on pathogen closely related to the acquisition of agrocine 84 resistance by the pathogen.

Competition

Once a BCA is applied in the host tissue, it might compete with pathogen and natural microbiota for either nutrients or space. If the BCA is more efficient to establish in the surface or to acquire nutrients from the environment than pathogen, the last will be displaced. This kind of mechanism has a preventive effect, inhibiting development of pathogen but cannot remove it. Bacteria and yeasts are microorganisms with a high capacity to biocontrol fungal diseases by means of

competition for nutrients. Their large surface-to-volume ratios allow to take nutrients from dilute solutions more rapidly and at greater quantity than the germ tubes of fungal pathogens (Fokkema *et al.*, 1981), being molecules in low concentration in fruit wounds, such as nitrogen compounds, can be critical during the competition process. Studies on the distribution of radiolabelled glucose between the antagonistic yeast *Sporobolomyces roseus* or *C. laurentii* and the pathogen *B. cinerea* show strong sugar consumption by the BCA that blocks conidial germination of the pathogen (Filonow, 1998). In case of bacteria, there are outstanding BCAs more competitive than the pathogen, for instance *Enterobacter cloacae* against *R. stolonifer* on peache (Wisniewski *et al.*, 1989), *Pseudomonas fluorescens* EPS62e against the bacterial pathogen *E. amylovora* on pear (Cabrefiga, 2004), *E. cloacae* against the fungus *Pythium ultimum* (van Dijk and Nelson, 2000), *Bacillus pumilus* 3PPE and *Bacillus amyloliquefaciens* 2TOE strains against grey mould on pear (Mari *et al.*, 1996), and finally, strains of *P. agglomerans* with different plant pathogens (Kempf and Wolf, 1989).

In the competition for space, the antagonist colonizes the infection site excluding the pathogen and inhibiting the development of disease. Thus, this kind of BCA has to be able to colonize and multiply more quickly than pathogen. In this way, *P. agglomerans* has a higher competitive ability than the phytopathogen *P. syringae* (Pasichnyk *et al.*, 2005). Moreover, it has been reported that the formation of an extracellular polysaccharide capsule by yeasts can promote adhesion of BCA to the fruit surface being more competitive than pathogen (Andrews *et al.*, 1994).

Induced resistance in the host tissue

Induced resistance in the host is a mechanism triggered by some microorganisms or chemical agents by which chemical and physical barriers are induced providing a systemic protection against a broad range of pathogens. When the elicitation of this systemic protection is mediated by non-pathogenic aerial colonizators it is called Systemic Acquired Resistance (SAR) but whether it is mediated by rhizobacteria is known as Induced Systemic Resistance (ISR) (van Loon *et al.*, 1998).

In postharvest, some BCAs can interact with wounds increasing the cicatrisation processes favouring antagonist settlement (Mclaughlin *et al.*, 1990; Droby and Chalutz, 1994). Concretely, El Ghaouth *et al.* (2001) have proved that wounds treated with *C. saitoana* induced a gradual systemic resistance against the pathogen *B. cinerea* in apple, which was related chitinases and β-1,3-glucanases levels. In addition, it has been found several aromatic compounds, essential oils and volatile substances produced by several microorganisms, such as 1-methylcyclopropene, that delay this process in apple (Janisiewicz *et al.*, 2003). A strain of *Pichia guilliermondi* stimulates the production of ethylene on grapefruit, a hormone that activates phenylalaninammonium-lyase, an enzyme involved in the synthesis of phenols, phytoalexins and lignins (Wisniewski *et al.*, 1991), Moreover, it is possible to reach a synergistic effect through the application of the combination of BCA with antifungal compounds. For example, *C. laurentii* can be applied together with methyljasmonate to control brown rot and blue mould in peach fruit (Yao and Tian, 2005).

Direct interaction

Direct interaction is based on the ability of some BCAs to recognize and interact with the pathogen interfering disease development. So far, hyperparasitism is the kind of direct interaction most studied and has the aim to obtain nutrients for development. It occurs on several phases: (i) chemiotropic attraction of the BCA to the pathogen, (ii) recognition of pathogen receptors, (iii) growth and adhesion of the BCA along the hyphae of pathogen and (iv) production of lytic enzymes such as chitinases, glucosidases and celulases (Handelsman and Parke, 1979) to degrade the fungal cellular wall (Elad *et al.*, 1982; Sivan and Chet, 1992; Lorito *et al.*, 1993).

There are several BCA able to parasite other fungal pathogens such as fungi *Trichoderma harzianum* (Elad *et al.*, 1982), bacteria with chitinolitic activity as for example *P. fluorescens* (Nielsen

and Sorensen, 1999) or *P. agglomerans* (Chernin *et al.*, 1995) and yeasts as *Aureobasidium pullullans* that produce extracellular chitinase and β-1,3-glucanase in apple wounds (Castoria *et al.*, 2001).

Quorum quenching

Quorum sensing controls a range of activities implicated in pathogen-host interaction and microbe-microbe competition by means of *N*-Acyl homoserine lactones (AHLs) signals, such as virulence genes (Pirhonen *et al.*, 1993; Passador *et al.*, 1993; Jones *et al.*, 1993) and production of antibiotics (Bainton *et al.*, 1992; Holden *et al.*, 1998). For this reason it is suspected that it may provide a competitive advantage in natural environment to quorum sensing bacteria with regard to other occurring microorganisms. Some microorganisms have developed a signal interference strategy to counteract the quorum sensing signalling of their competitors named quorum quenching (Dong *et al.*, 2001; Bauer and Robinson, 2002; Zhang, 2003). Two groups of AHL-degrading enzymes produced by several soil bacterial species have been described in bibliography, AHL-lactonases of *Bacillus* strains and AHL-acylases of *Ralstonia* and *Variovorax paradoxus* (Dong *et al.*, 2000; Lee *et al.*, 2002; Dong *et al.*, 2002; Lin *et al.*, 2003). Specifically, a study performed by Dong *et al.* (2004) showed that *Bacillus thuringiensis* significantly decreased the incidence of *Erwinia carotovora* infection *in planta* through production of AHL-lactonases. Another report showed that *Bacillus* sp. strain A24 with AHL-lactonase activity provided significant preventive and curative biocontrol against the potato soft rot caused by *E. carotovora* and crown gall of tomato incited by *Agrobacterium tumefaciens* (Molina *et al.*, 2003).

In relation with the mechanism that conferred the biocontrol capacity to strain EPS125, it seems that the main mechanism of biocontrol is not antibiosis as show results obtained by *in vitro* inhibition assays performed in cocultivation with *E. amylovora*, *P. syringae*, *P. expansum* and *S. vesicarium* in several culture media; and with *M. laxa* and *R. stolonifer* on peel leachate and nectarine juice. An interesting result to commercialize this biopesticide as it has not the negative effects of antibiotics use. In addition, EPS125 has shown to be able to inhibit conidia germination of *M. laxa* and *R. stolonifer* by means direct cell-to-cell interaction, without intervention of parasitism interaction or nutrient competition (Bonaterra *et al.*, 2003). In conclusion, preemptive exclusion by wound colonization and direct interaction with the pathogen have been proposed as the main mechanism of biocontrol of EPS125 to these fungus. However, it is not known in the case of *P. expansum*.

As it has been commented above, to determine the mechanism of action in a biocontrol agent is very difficult because there are several mechanisms involved and there are not enough tools to study them in an independent way. Different approaches have been performed to find the mechanism employed by BCAs such as *in vitro* inhibition assays for antibiosis, comparison of growth corves for nutrient competition or observation of the BCA and the pathogen by microscopic techniques for direct interaction or colonization studies. However, to demonstrate what mechanisms are responsible of the biocontrol activity, phenotypic approaches have to be accompanied by genetic studies.

There are several molecular tools to detect gene or genes implied in pathogenic and biocontrol activity; however, mutation of essential genes responsible of these abilities has been the most broadly used. Amongst mutation methods described in bibliography, mutagenesis with transposons is the most used suitable tool to perform this kind of analysis as it allows to generate mutations in target sequences (target-selected mutagenesis), as well as in unknown random genomic sequences. This last property is interesting to determine unknown aspects of genome function such as genes implied in unknown biocontrol mechanisms. A large range of transposons have been reported, of which several variants have been developed from Tn5 transposon according to marker selection, gene reporter, specificity of insertion, size and polarity (Handfield and Levesque, 1999) as for instance the minitransposon GUS (mTn5SS*gusA40*) (Wilson *et al.*, 1995). This minitransposon carries genes that led resistance to spectinomycin and

streptomycin antibiotics and a GUS reporter gene that allows the study of regulation of gene expression. Therefore, owing to absence of GUS activity in plants and many bacteria, this transposon represents a powerful tool for studying the ecology of bacteria and interactions occur on plants in field conditions.

However, the use of mutagenesis with transposons to determine the biocontrol mechanisms used by any BCA involves some drawbacks that are unlikely to be overcome. In first place, it has to develop a sensible screening method with low variability of results that allows the detection of true biocontrol defective mutants. Another limitation of the mutagenesis technique is that it cannot rule out pleiotropic effects resulting from the disruption of specific genes. In addition, sequences disrupted by the transposon insertion might not have a known function or described on bibliography obstructing its analysis. Finally, it is possible that the total loss of biocontrol activity was not reached because of other biocontrol mechanism may be involved. However, in spite of these limitations, mutagenesis with transposons is the best option to elucidate which biocontrol mechanisms are employed by any BCA with unknown DNA genomic sequence.

Register

Despite the efforts performed to develop commercial biocontrol products, only a few have been registered in the European Union. The register of plant protection products is regulated by the Directive 91/414/EEC, which was amended specifically for biopesticides by the Directive 2001/36/EC. This directive requests identification and characterization of the microorganism until strain level, determine biological properties to perform toxicological studies, and develop techniques for traceability and environmental impact studies. Most companies are not attracted for this kind of products because of the difficulty to register a product, it shows reduced range of application and supposes an important economical risk for manufactures. Table 3 shows some biopesticides actually registered or under development in the world, as is the case of *P. agglomerans* D325 named "Bloomtime Biological" (Northwest Agricultural Products, Pasco, WA)(Pusey and Curry, 2004), which inhibits the pathogen *E. amylovora*.

As it has stated above, the biological control agent EPS125 isolated in our laboratory is highly effective in the control of several postharvest diseases on different types of fruit (Bonaterra *et al.*, 2003; Francés *et al.*, 2006). This strain is deposited in the Spanish Type Culture Collection as *Pantoea agglomerans* CECT5392 and is the object of a patent with the reference number EP1 402779A1. In addition, a mass production system is available for the biocontrol strain EPS125, as well as a formulation based on osmotic induction that improves its survival ability (Bonaterra *et al.*, 2005). In relation with mechanism of biocontrol used by EPS125 to inhibit fungal pathogens, antibiosis as mainly biocontrol mechanism has been discarded and direct cell-to-cell interaction and preemptive exclusion of niche seem to be involved (Bonaterra *et al.*, 2003).

For all these reasons, EPS125 is considered an interesting candidate to be developed as a commercial microbial fungicide for postharvest control. However, to register and commercialize this biopesticide it is necessary to perform a careful identification and an extensive characterization of the strain, as well as exhaustive studies about the mechanism of action of EPS125 against *P. expansum* in apple fruit by means of phenotypic and genotypic studies.

Table 3. Products registered or under development for biocontrol of plant and postharvest diseases in USA and other countries (http://www.epa.gov/pesticides/biopesticides/product_lists/index.htm)

Active ingredient	Product name	Target Pathogen
A. radiobacter	Nogall Galltrol-A	*A. tumefaciens* *Agrobacterium rhizogenes*
B. subtilis GBO3	Kodiak	*Rhizoctonia* *Fusarium*, *Aspergillus* and others
Bacillus thuringiensis	Several	Many insects
C. oleophila I-182	Aspire	*Botrytis* *Penicillium*
Coniothyrium minitans CON/M/91-08	Contans WG	*S. sclerotiorum* *Sclerotinia minor*
Fusarium oxysporum	Biofox C	*F. oxysporum* *Fusarium moniliforme*
Gliocladium virens GL-21	WRC-AP-1	*Ralstonia solani* *Phytium*
Myrothecium verrucaria	DITERA DF Biological Nematode	Parasitic nematodes
P. agglomerans D325	Bloomtime Biological Bloomtime Biological FD	*E. amylovora*
Phitium oligandrum	Polygandron	*P. ultimum*
Pseudomonas aureofaciens Tx-1	STOP-LESS Biofungicide	*Sclerotinia homeocarpa* *Collectotrichum graminicola* *Pythium aphanadermatum* *Michrodochium nivale*
Pseudomonas chlororaphis 63-28	ATEZE	*Pythium* spp. *R. solani* *F. oxysporum*
P. fluorescens A506	FROSTBAN A FROSTBAN B BlightBan	*E. amylovora*
P. syringae ESC 10 and ESC11	Biosave	*B. cinerea* *Penicillium* *Mucor pyroformis* *Geotrichum candidum*
Pseudozyma flocculosa PF-A22 UL	SPORODEX L biological fungicide	Powdery mildew
T. harzianum Rifai T-39	T-22 Planter Box Biological Fungicide FAFARD Growing Mix With Rootshield Granules T-22G Biological Plant Protectant Granules	*B. cinerea*

3. Objectives of the PhD

The main objectives of this work were:

1. Identification and characterization of strain EPS125 by means of phenotypic and genotypic methods.
2. Development of a specific monitoring for EPS125 based on intrinsic DNA molecular markers.
3. Determination of the mechanism of action used by strain EPS125 against the postharvest pathogen *P. expansum* on apple through phenotypic approaches and genetic analysis using mutagenesis with transposons.

Chapter I
Characterization of *Pantoea agglomerans* EPS125

1. Introduction

Biological control agents (BCAs) are an increasingly important alternative or complement to chemicals in crop and postharvest protection. Its commercial use involves a multitiered process that includes selection, identification, efficacy trials, toxicology testing, mass production, formulation and register (Woodhead *et al.*, 1990; Cross and Polonenko, 1996). Specifically, the register of a biopesticide requires a precise ascertainment of its identity and stability, which is of paramount importance to ensure efficacy and genetic integrity of the product and to protect intellectual property.

Several strains of *Pantoea agglomerans* (*Erwinia herbicola* or *Enterobacter agglomerans*) have been selected as BCAs against bacterial and fungal pathogens (Nelson, 1988; Vanneste *et al.*, 1992; Kempf *et al.*, 1993; Yuen *et al.*, 1994; Han *et al.*, 2000; Braun-Kiewnick *et al.*, 2000; Yuen *et al.*, 2001; Bonaterra *et al.*, 2003; Francés *et al.*, 2006) in spite of members of the complex *Erwinia* genus can cause diseases of plants. In fact, although the heterogeneous taxonomic structure of the genus *Erwinia* has been discussed by using phenotypic data (Dye, 1968; Dye, 1969a; Dye, 1969b; Ewing and Fife, 1972; Gardner and Kado, 1972; Dickey, 1979; Dye, 1981; Thomson *et al.*, 1981; Mergaert *et al.*, 1984; Verdonck *et al.*, 1987) and genotypic data (Brenner *et al.*, 1972; Brenner *et al.*, 1973; Brenner *et al.*, 1974; Brenner *et al.*, 1977; Azad and Kado, 1980; Brenner *et al.*, 1984), the taxonomic position of this genus remains still problematic (Starr and Chatterj, 1972; Thomson *et al.*, 1981; Brenner *et al.*, 1984; Lelliott and Dickey, 1984). Therefore, owing to heterogeneity and complexity of this group, an accurate identification and characterization including toxicological tests of *P. agglomerans* strains by means of phenotypic and genotypic methods are indispensable to develop them as biopesticides.

In principle, phenotypic and genotypic information are collected to perform a polyphasic analysis with the aim to identify a target microorganism or to determine phylogenetic relationships between several microorganisms (Figure 1.1). Phenotypic methods analyses different traits of bacteria including: (i) protein content by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); (ii) chemotaxonomic markers such as cell wall composition, cellular fatty acids (FAME), isoprenoid quinones from cytoplasmic membrane, polyamines and production of exopolisaccharides; and (iii) a wide range of other expressed features based on the study of morphology, physiology, enzymology and serology of cells (Vandamme *et al.*, 1996). However, results from traditional phenotypic identification might be

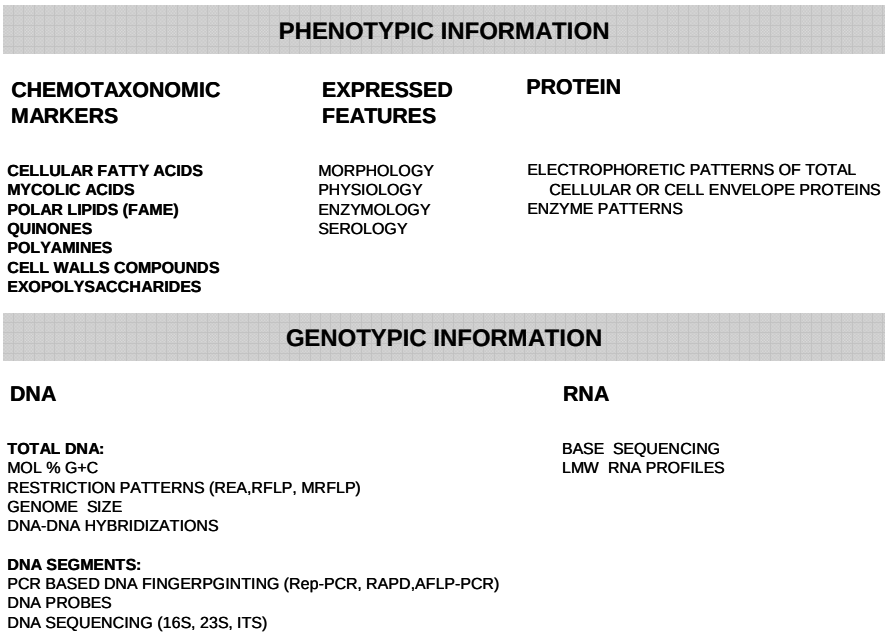

Figure. 1.1 Phenotypic and genotypic traits used during polyphasic analysis (Vandamme *et al.*, 1996).

influenced by growth conditions and for many slow growing organisms, being a difficult and time-consuming process.

Genotypic methods are those that are directed toward DNA or RNA molecules, and are emerging as an alternative or complement to established phenotypic methods offering a variety of advantages over more conventional methods. In the first place, genotypic methods are based on stable characteristics of the genetic information contained within bacteria avoiding problems of phenotypic plasticity related with growth conditions. In addition, measurement or determination of a bacterial genotype does not require growth of the bacterium in the laboratory making possible the identification of bacteria without its growth. Moreover, genotypic characterization and identification of most bacteria is more rapid and sensitive than phenotypic methods (Vandamme *et al.*, 1996).

There are two established groups of genotypic characterization methods, independent-PCR methods and PCR-based methods. The first group comprises the following methods: (i) determination of the moles percent guanosine plus cytosine (G+C); (ii) DNA-DNA hybridization studies that provide indirect parameters of the sequence similarity between two entire genomes; (iii) studies of homology of rRNA molecules (16S and 23S), which are the best target for studying phylogenetic relationships because they are present in all bacteria, are functionally constant, and are composed of highly conserved as well as more variable domains (Woese, 1987; Schleifer and Ludwig, 1989; Stackebrandt and Goebel, 1994); (iv) comparison of plasmid profiles; and (v) typing methods based on the analysis of whole-genome restriction fragment such as Restriction Endonuclease Analysis (REA), Restriction Fragment Length Polymorphism (RFLP), or Macrorestriction Fragment Length Polymorphism (MRFLP) (Vandamme *et al.*, 1996; Badosa, 2003). However, to perform this kind of genotypic characterization, large amounts of DNA are indispensable.

PCR methodology arises as a simple and rapid alternative to independent-PCR techniques and offers a vast array of applications related to genotypic characterization. Specifically, within PCR-based methods group there are several strategies that analyse the genome structure without previous knowledge of their nucleotide sequence. For example, RAPD (Random Amplified Polymorphic DNA) and RAPD-related methods that obtain

characteristic band patterns by using short primers of arbitrary sequence; and rep-PCR method that analyses length polymorphism amplified fragments using DNA primers corresponding to interspersed repetitive elements sequences such as REP-PCR (Repetitive Extragenic Palindromic sequence of 35-40 bp), ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus sequence of 124-127 bp) and BOX-PCR (BOX conserved element of 154 bp) (Versalovic *et al.*, 1991).

In addition, the use of PCR technique combined with restriction analysis has been described as well. For instance, the AFLP-PCR (Amplified Fragment Length Polymorphism-PCR) method, which is based on restriction fragment length polymorphism analysis followed by ligation of short oligonucleotides (or adapters) and PCR amplification (Yang *et al.*, 1993).

Some of these genotypic methods not only have been employed for identification and characterization of microorganisms, but also they have been suitable for monitoring of specific BCAs. For example, Pujol *et al.* (2005) have developed a molecular marker known as SCAR (Sequence Characterized Amplified Regions) for the biocontrol agent *Pseudomonas fluorescens* EPS62e of fire blight from one discriminatory fragment of its RAPD profile. Nowadays, DNA markers based on specific amplification of a target DNA sequence that is uniquely present in a determinate BCA are developing for monitoring biopesticides allowing their unambiguous identification and quantification in field conditions.

All studies accomplished with the biocontrol agent EPS125 point to as an interesting candidate to be developed as a commercial microbial fungicide for postharvest control. However, an intense characterization and identification of strain EPS125, a molecular monitoring method which allows the specific detection of the biopesticide EPS125, as well as effectiveness study of EPS125 against *P. expansum* on apple fruit have not been performed yet.

2. Objectives

The aims of this chapter were to perform an identification and characterization of the strain EPS125 at phenotypic and genotypic levels, as well as to develop a molecular tool for it specific detection. Moreover, the study of effectiveness of EPS125 against *P. expansum* on apple fruit by means of dose-response assays was included in this chapter

3. Materials and Methods

The strain EPS125 has been characterized and identified by means of phenotypic and genotypic tests in comparison with other strains from INTEA and CECT strain collections. For long term preservation, these strains were stored with 20% glycerol at -80 ºC under deep-freeze (Sanyo, Tokyo, Japan).

Composition of media, buffers and reagents used in this work are detailed in annex I section.

3.1. Phenotypic characterization

3.1.1. Biochemical and morphological tests

The first approach used to identify the strain EPS125 was by means of classical biochemical and morphological tests that are shown in Table 2.1.

Table 2.1. Morphological and biochemical tests used to identify strain EPS125.

Test	Reagent	Detection	Reference
Morphology	-	Observation by Optical Microscopy	-
O/F	Medium O/F	Turn to yellow colour	Hugh and Leifson, 1953
Oxidase	tetramethyl-p-phenylenediamine	Turn to blue colour	Kovaks, 1956
Pigmentation	-	Observation	-
Gram stain	Ryu potasse (KOH 3 %)	Solubility	Suslow *et al.*, 1982
Voges-Proskauer	α–naftol; KOH 40%	Reddish-violet colour	Schaad, 1988

In addition, an Analytical Profile Index (API) 20E (BioMérieux, Lyon, France) strip, which is appropriate to identify enterobacteria, was inoculated with a single colony suspension of the strain EPS125 following indications of the supplier (Figure 3.1). This strip contained 20 standardized biochemical tests: β-galactosidase, arginine dihydrolase, lysine descarboxylase, ornithine descarboxylase, citrate utilization, H_2S production, urease, tryptophan deaminase, indole production, acetoin production, gelatinase, glucose utilization, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose fermentation or oxidation. Strips were incubated at 25 ºC for 48 h. Identification of the strain EPS125 was performed by means of API20E analytic catalogue (Catalogue analytic API 20E, 1985).

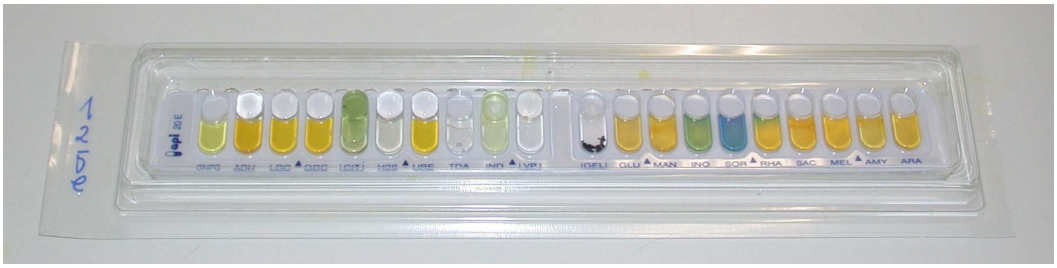

Figure 3.1. API 20E strip of strain EPS125.

3.1.2. Hypersensitive Response (HR) in non-host plant

HR test is based on the capacity of non-host plants to respond against phytopathogens triggering a visible hypersensitive response (HR) (Klement, 1963). In this work, tobacco plant (*Nicotiana tabacum*) was selected as model plant of cv. Xanthi to perform the pathogenicity test.

Using a syringe, leaves of young tobacco plant were transversely wounded between ribs. Getting blocked way out of wound with a finger; wounds were inoculated by the underside of leaves with a suspension of the EPS125 strain adjusted turbidimetrically to 10^8 cfu/ml, using a syringe without needle. Similarly, a positive control was carried out with the pathogenic strain *P. syringae* EPS94 and a negative control with sterile distilled water. After inoculation, plants were incubated during 48 h at 20 ºC with 80 % of relative humidity in a Sanyo Growth cabinet (Sanyo, Tokyo, Japan).

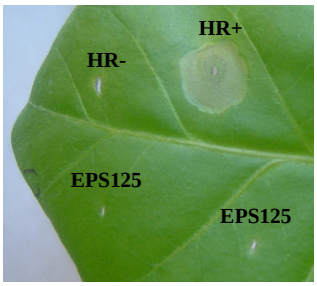

Figure 3.2. HR expressed on Tobacco plant. **HR+**: Positive control. **HR-**: Negative control. **EPS125**: Strain EPS125.

The response was considered positive when the inoculated strain led to a blocked necrosis limited to the infiltration zone between two ribs showing dried tissue of beige greyish colour and metallic shine (Klement, 1963). Response was negative when the strains did not promote any alteration in the plant tissue (Figure 3.2).

3.1.3. Ice Nucleation Activity (INA)

Some bacteria show ice nucleation activity due to the synthesis of specific external membrane proteins, which produces freezing of water at a higher temperature than usual and it may promote frost damage to plant tissues (Lindow *et al.*, 1983).

To detect if strain EPS125 has ice nucleation activity, a suspension adjusted at 10^8 cfu/ml was incubated for 1-2 h at 0-4 ºC in a refrigerator. After it was incubated at a temperature gradient from 0 to -10 ºC for 20 min in a B8-30E antifreeze cryogenic bath (Heto, Allerod, Denmark). A positive control with *Pseudomonas syringae* EPS94 and a negative control with sterile distilled water were included in this assay.

Strains were positive for INA when the suspension was frozen in 20 min and negative when it did not freeze.

3.1.4. Utilization of carbon sources

Strains EPS125, EPS135, EPS403, EPS417, EPS452, EPS468, EPS482, EPS494, EPS495, EPS501, EPS513, EPS528, EPS209 and EPS550 were characterized according to the utilization of 95 carbon sources using Biolog GN microplate (Biolog Inc, Hayward, USA). Cultures were grown on BUGTM agar (Biolog Inc, Hayward, USA) for 24 h at 22 ºC. Bacterial suspensions adjusted at 4.5 x 10^8 cfu/ml were prepared in saline solution (0.85 %) and were inoculated in plates according to manufacturer indications. Microplates inoculated with *Pseudomonas* suspensions were incubated for 24 h at 28 ºC, whereas those inoculated with *Erwinia* were incubated for 6 h at the same temperature. After incubation period, optical density of each well was read at wavelength of 405 nm using a multiscan photometer (Labsystems Multiskan RC, Barcelona, Spain) operated with the ASCENT software (Ascent Research Edition Version 2.1, Labsystems, Helsinki, Finland). Equal or higher absorbance values than 0.2 were considerate as positives.

These results allowed to calculate similarity indexes between different strains by means of the Sokal coefficient (Sneath and Johnson, 1973). With the obtained similarity values, a cluster analysis was performed using the statistical program SPSS for Windows (version 7.5.2 S. SPSS. Ind. 1989-1997). By means of the agglomerative hierarchical grouping method and through the simple correlation method, a dendrogram was obtained where strains were grouped based on similarity percentage of utilization of several carbon sources.

3.1.5. Fatty Acid Methyl Ester (FAME) profile

Bacterial cell membrane is constituted by a large diversity of hydrocarbonated chains with variable length and structure, but stable in a same taxon. FAME profile has become a useful tool to identify and characterize several groups of microorganisms. Up to now, this analysis has been a standard test to identify anaerobic bacteria (Holdeman *et al.*, 1977), and a growing body of literature suggests its use to identify aerobic bacteria (Moss and Dees, 1975; Dees and Moss, 1975; Drucker *et al.*, 1976; Moss, 1981).

Strains EPS125, EPS403, EPS417, EPS452, EPS468, EPS482, EPS494, EPS495, EPS501, EPS513, EPS528 and EPS550 were grown on Tryptone Soya B Agar (TSBA) for 24 h at 28 ºC. After the incubation period, bacterial mass was collected in a glass tube and was processed according to the modified method of Miller and Berger (1985). In first place, a saponification of fatty acids was performed by addition of 2 ml of a NaOH-MetOH solution (reactive 1, annex I) to each tube. Suspensions were briefly stirred with a Genie-2 vortex (Scientific industries Inc., NY, USA) and were incubated for 5 min at 100 ºC in a bath (Selecta, Barcelona, Spain). Samples were vigorously mixed again and tubes were incubated for 25 min at 100 ºC. Metilation was accomplished adding 4 ml of an HCl-MetOH solution (reactive 2, annex I) to each tube. All samples were stirred and were incubated for 10 min at 80 ºC (± 1 ºC). Finally, an extraction step was performed by addition of 1.5 ml of a reactive 3 (annex I) to each tube. Samples were mixed for 10 min by continue inversion with a Rotamix 1 shaker (Iberlabo, Madrid, Spain) and were rested upright until two solution phases separated. The watery phase (lower phase) of each tube was discarded and 3 ml of a saturated NaCl solution (Reactive 4, annex I) were added. Samples were mixed for 10 min by continue inversion and were rested upright for 5 min. The top phase containing fatty acids were collected in chromatography vials and stored at room temperature in case of they were analyzed before two weeks or at 4 ºC for long term storage.

Chromatography analysis was performed in the Laboratori del Servei de Protecció Vegetal del Departament d'Agricultura de Ramaderia i Pesca (Barcelona, Spain) using a HP 5890 series II gas chromatograph (Hewlett Packard, Minnesota, USA) equipped with a Ultra2Crosslinked 5 % Ph Me Silicone column (25 m of long, 0.2 mm of outside thickness and 0.33 μm of diameter). The temperature program was ramped from 170 to 250 ºC at 5 ºC/min. The sample was injected by means an automatic injected HP7673A (Hewlett Packard, Minnesota, USA). The calibration standard used was supplied by Microbial Identification System (Part Nº: 19298-60500 Rev B. Agilent). Chromatograph analysis result was gathered through control software (HPGC. Chem. Station Rev. A. 07. 01 (682) HP. 1990-1999) and processed by means Sherlock ver. 3.10 program (Microbial Identification System, MIDI, Inc., 125 Sandy Drive, Netwark, DE19713, USA). With the processed results, which correspond to several fatty acid relative contents for each strain, Euclidean distances were calculated. Dendrogram was achieved by applying the Agglomerative Hierarchical group method and using SPSS 7.5 programme. In addition, Sherlock programme provided identity values for each analyzed strain regard to reference strains deposited in Microbial Identification Data programme. Identity values higher than 0.6 were considered as a correct identification.

3.1.6. Dose-response assays

Dose-response assays provide quantitative parameters that describe the pathogen virulence as well as the efficiency of the biocontrol agent permitting the comparison of different BCAs and pathosystems (Montesinos *et al.*, 1996; Larkin and Fravel, 1999; Bonaterra *et al.*, 2003). In the present work, two dose-response assays were performed in different periods of the year and using 'Golden Delicious' apple fruit of two different harvestings, one early in the summer and other at the end of the same summer, from orchards of Mas Badia Agricultural Station located in Girona. Fruits were free of wounds and rot and were homogeneous in maturity and size. Fruits were stored at 4 ºC.

Preparation of biocontrol agent and pathogen suspensions

The effect of pathogen and biological control agent concentrations on the severity of fruit rot was assessed. These were prepared as described section 2.1.2. Inoculum concentrations were $5x10^2$, 10^3, 10^4 and $5x10^4$ spores/ml for *P. expansum* and 0, 10^5, 10^6, 10^7, 10^8 and 10^9 cfu/ml for *P. agglomerans* EPS125.

Preparation of the plant material

Apple fruit were surface-disinfected by immersion for 10 min in a dilute solution of sodium hypochlorite (1% active chlorine), washed twice by immersion for 10 min in distilled water and a final immersion for 10 min in sterile distilled water, and left to air dry. Then, they were wounded in the middle of the equatorial zone with a cork borer making a single wound per fruit of approximately 6 mm^2 and 5 mm depth.

Inoculation and incubation of the plant material

Wounds were inoculated with 50 µl of the antagonist suspension, let stand for 1 h for complete water absorption within wound, and inoculated with 10 µl of the spore suspension of the pathogen. Then, fruit were placed in polystyrene tray packs in boxes that were sealed with plastic bags to maintain high humidity and incubated at 15 ºC in a controlled environment chamber (PGR15, Conviron, Canada).

The experimental design consisted of 3 replicates of 5 fruit per replicate. The replicates were completely randomized within the incubation chamber.

Analysis of results

Diameter of each lesion was determined after 5 days inoculation. Disease severity for each apple was calculated as the diameter of lesion expressed as a proportion of the highest diameter value obtained in the non-treated controls. These disease severity data were used to estimate efficiency parameters for the biocontrol agent and pathogen using a hyperbolic saturation model (HS) (Montesinos *et al.*, 1996). The equation of the hyperbolic saturation model which relates disease to concentrations of the biocontrol agent and pathogen is as follows:

$$y = Y_{\max} \frac{x(1-I)}{x(1-I)+K_x}$$

Where Y_{max} is the maximum disease proportion the pathogen can produce, K_x a half-saturation constant corresponding to the pathogen concentration producing half the maximum disease proportion, x the pathogen density and I is the proportion of the pathogen inactivated by the biocontrol agent concentration. The proportion of inactivated pathogen (I) depends on the biocontrol agent concentration (z) according to the following equation:

$$I = I_{\max} \frac{z}{z+K_z}$$

Where I_{max} is the maximum proportion of pathogen the biocontrol agent can inactivate and K_z is the concentration of biocontrol agent that produces an inactivation of I_{max} /2.

This model provides valuable parameters such as the median effective dose (ED_{50}) of the pathogen (K_x) and the biocontrol agent (K_z) and the efficiency of the biocontrol agent calculated as the ED_{50} biocontrol agent/pathogen ratio (K_z/ K_x).

Regression and parameter estimation were performed by a non-linear-least-squares method using the NLIN procedure of the SAS (SAS Institute Inc, 1993). One of the parameters (e. g. Y_{max}) was fixed, and a new set of iterations performed to find a first solution. Then, the new parameters values obtained were used as initial values for a new iteration until a likelihood solution was obtained. The goodness of fit was evaluated according to the mean square error (MSE) for the model and the asymptotic standard errors (ASE) for the parameters.

3.1.7. Complementary tests

Production of Indol Acetic Acid (IAA)
A nitrocellulose membrane filter was deposited on plates containing IAA medium (annex I). Using a sterile toothpick, the strain EPS125 and *P. fluorescens* CHAO as positive control were spotted in plates containing IAA medium. After 3 days of incubation at 28 ºC, a Whatman 2 filter paper saturated of Salkowski reagent (annex I) was deposited on plates. Strains were positive IAA when the colonies appeared pink-red colored after 30 min to 3 h of treatment. Result was negative when it presented a yellowish-brown colour (Bric *et al.*, 1991).

Sensitivity to antibiotics
In later stages of the present work it was interesting to test the sensitivity of the strain EPS215 on LB medium supplemented with the following antibiotics: ampicillin (100 μg/ml), carbenicillin (100 μg/ml), chloramphenicol (25 μg/ml), spectinomycin (25 μg/ml), streptomycin (10 μg/ml), gentamicin (50 μg/ml), kanamycin (100 μg/ml), rifampicin (50 μg/ml) and tetracycline (25 μg/ml).

3.2. Genotypic characterization

3.2.1. Sequencing of 16S rDNA

Ribosomes are essential to synthesize proteins and the genes coding for ribosome RNA are interesting for genotypic characterization of strains because these remain unalterable to environmental changes. Currently, they are a useful tool to identify and study phylogenetic relationships of microorganisms because accumulated changes during ribosome evolution are considered equivalent to the accumulated changes in the genome. The rRNA molecules have two regions, an essential conserved domain and variable sequences that increase the phylogenetic resolution at species or subspecies levels (Gutell *et al.*, 1994; Head *et al.*, 1998).

DNA extraction
DNA of strain EPS125 and two strains identified as *P. agglomerans* from CECT collection, CECT4842 and CECT850T, were extracted following a modified protocol of Keel *et al.* (1996) and described by Badosa (2003).

One isolated colony was inoculated in 10 ml of Luria Bertrani (LB) broth. Once culture was grown for 24 h at 25 ºC with continuous shaking at 125 rpm in a KS501 digital orbital shaker (IKA Labortechnik, Staufen, Germany), 10 μl of culture were transferred to a tube containing 90 μl of lysis buffer rDNA (annex I). Later, tube was incubated for 10 min at 99 ºC in

a thermobloc multibloc (Selecta, Barcelona, Spain). It was used inmediatly or stored at -20 ºC until its use.

Amplification

Primers used for the amplification of 16S rDNA gene were 8f and 1492r (Gotz *et al*., 2002) (Table 3.2). Amplification reaction was performed in a total volume of 50 µl containing 1X buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 µM of each oligonucleotide (Roche diagnostics, Manheim, Germany), 1.5 U of *Taq* DNA polymerase (Invitrogen Life technologies, California, USA) and 5 µl of DNA extraction. Amplification reactions were performed with the Gene Amp ® PCR System 9700 thermal cycler (PE Applied Biosystems, MA, USA). Thermocycle conditions consisted of an initial denaturation at 95 ºC for 2 min followed by 30 cycles of 95 ºC for 60 s, 52 ºC for 60 s, 72 ºC for 90 s, and a final elongation at 72 ºC for 5 min.

PCR products and 1 Kb plus ladder marker at 50 ng/µl (Invitrogen Life technologies, California, USA) were separated by horizontal electrophoresis with a Mini-sub®Cell (BioRad, California, USA) on a 1.5 % agarose gel in 1X TAE buffer (annex I) at 65 V for 45 min. After this time, gel was stained with ethidium bromide (1 µg/µl) for 30 min. The PCR products were viewed with a UV transilluminator FX-20M (Vilvert Lournat, France). Pictures were digitally captured with a digital camera DC120 (Kodak, Madrid, Spain) connected to a computer and their processing were performed with the 1D Image analysis system 120 software (Kodak, Madrid, Spain). Fragment sizes were calculated by means the 1D Image analysis system 120 software.

Sequencing

To avoid the presence of unspecific fragments during sequenciation reaction of 16S rDNA gene, the amplified 16S rDNA was recovered from agarose gel and cleaned with Qiaex II Handbook kit (Qiagen GmbH, Hilden, Germany) according to manufacturer' indications. Sequencing reactions of the amplified products obtained of strains EPS125, CECT850 and CECT4842, were performed with the BigDye Terminator v3.0 Ready Reaction cycle sequencing kit (Applied Biosystems, California, USA). Each sequencing reaction was performed in a total volume of 10 µl containing 1 µM of primer, 3 µl of Terminator Ready Reaction Mix and 40 ng of PCR product. In table 2.2 are detailed all primers used to sequence the amplified fragment of 16S rDNA. Primers developed in this work were designed using Primer Express™ software (PE Applied Biosystems, MA, USA) from known sequences.

Table 3.2. Sequences of primers used to amplify and sequence 16S rDNA of strain EPS125.

Primers	Sequence (5'-3')	Reference
7f	AGA GTT TGA TCC TGG CTC AG	Iwagami *et al*., 2000
8f	AGT TTG ATC CTG GCT CAG	Gardener and Weller, 2001; Hayes and Lovley, 2002; Gotz *et al*., 2002
757f	GAT ACC CTG GTA GTC CA	This work
907r	CCG TCA ATT CCT TTG AGT TT	Hayes and Lovley, 2002; Gotz *et al*., 2002
1201r	GTA TGC GCC ATT GTA GCA	This work
1300r	TGG CAC GTA GTT AGC CGT	This work
1492r	ACG GTT ACC TTG TTA CGA CTT	Gardener and Weller, 2001; Hayes and Lovley, 2002; Gotz *et al*., 2002

r: reverse; f: forward.

Thermocycle conditions consisted of an initial denaturation at 94 ºC for 3 min followed by 25 cycles of 96 ºC for 10 s, 50 ºC for 5 s and 60 ºC for 4 min.

Amplified products were purified by means Ethanol/EDTA/Sodium acetate precipitation method described by Applied Biosystems. Samples were analyzed with ABI PRISM™ 310 Genetic Analyzer (PE Applied BioSystems, CA, USA) of the Molecular Biology Service of the Research Integrated Services of the Universitat de Girona (Girona, Spain).

Sequence analysis

The sequences obtained for each primer were aligned using Multialin program (Corpet, 1988), and were corrected manually by means of their chromatograms.

For a first identification of the three obtained sequences, these were introduced in the GenBank database by means of BLAST option (http://www.ncbi.nlm.nih.gov/BLAST/) (Madden *et al.*, 1996).

In addition, sequences were compared with the 16S sequence of *P. fluorescens* 62e (Pujol, M. personal communication) and other sequences of microorganisms related to *E. herbicola- P. agglomerans- E. agglomerans* deposited in GenBank (Table 3.3).

With the overall sequences, an analysis based on distances calculated with Kimura index of 2 parameters was performed using the Mega 2.0 program. Index values were used to construct a binary matrix which allowed draw the phylogenetic tree by means the Neighbour-Joining method. The strength was evaluated using a bootstrap resampling of 1000.

Table 3.3. Bacteria and access code of the 16S rDNA sequences obtained from the GenBank database.

Strain	Access code
Enterobacter agglomerans	AF130924
Enterobacter agglomerans	AF130927
Enterobacter agglomerans	AF130930
Enterobacter agglomerans	AF130933
Enterobacter agglomerans	AF130934
Enterobacter agglomerans	AF130937
Enterobacter agglomerans	AF130939
Enterobacter agglomerans	AF130944
Enterobacter agglomerans	AF130948
Enterobacter agglomerans	AF130949
Enterobacter agglomerans	AF130952
Erwinia amylovora	AJ010485
Erwinia carotovora	Z96091
Erwinia carotovora	Z96083
Erwinia herbicola	AB004757
Erwinia herbicola	U80202
Escherichia coli	X80730
Mycobacterium avium	AF306455
Mycobacterium ulcerans	X58954
Pantoea agglomerans	AF130895
Pantoea agglomerans	AF373196
Pantoea agglomerans	AJ233423
Pantoea aglomerans	AJ251466

3.2.2. Macrorestriction Fragment Length Polymorphism analysis (MRFLP)

Analysis of genomic macrorestriction fragments, which were obtained by digestion with enzymes of low frequency of cut and separated by pulsed Field Gel Electrophoresis (PFGE) is a useful tool to class bacteria to strain level (Badosa, 2003).

In this section DNA from strain EPS125, other strains belonging to *E. herbicola* species (EPS131, EPS512, EPS454 and EPS549), and several reisolates of strain EPS125 recovered from apples that were treated with this strain in previous assays, were extracted, digested and analyzed by PFGE.

One isolated colony was transferred to 6 ml of LB broth and cultures were incubated for 14 h at 27 ºC with slow shaking (100 rpm) in a KS501 digital orbital shaker. Bacterial suspensions were adjusted until an optical density of 0.6 at 600 nm with a spectrophotometer UV-160A. Then, 5 ml of each culture were dispensed in sterile centrifuge

tubes, which were previously cleaned with 96 % ethanol and were centrifuged at 14000 rpm for 15 min with Sorval RC 5C centrifuge (Plus Dupont, Connecticut, USA). Supernatants were discarded and bacterial cells were suspended with 5 ml of SEP buffer (annex I). The wash centrifugations were repeated three times more and pellets obtained were suspended in 0.5 ml of SEP buffer. Meanwhile, low point of fusion InCert®Agarose (FMC®Bioproducts, Maine, USA) was prepared at 1.6 % in SEP buffer and was maintained at 50 ºC in a UE300 culture heater (Memmert, Schwabach, Germany). Same volumes of bacterial suspension and 1.6 % agarose were mixed having into account for each plug was needed a volume of 100-110 µl. Mixtures were pipetted into plastic moulds that have been previously cleaned with ethanol and plugs were cooled down at room temperature during 1-2 h or at 4 ºC for 30 min. Below, cellular digestion was performed according to the method described by Zhang and Geider (1997). Solidified agarose plugs were transferred to a digestion solution containing 1 ml of buffer and 1 mg/ml of proteinase k (Sigma, Misouri, USA) and were incubated at 50 ºC for 72 h. Then plugs were cleaned by soaking in phosphate buffer (1 ml phosphate buffer/plug) (annex I) for 30 min at room temperature with slow shaking (125 rpm) in KS501 orbital shaker. Plugs were washed from 3 to 6 times more depending on the restriction enzyme used later. One third of plug was immersed in 500 µl of H buffer, restriction enzyme buffer supplied with the *XbaI* enzyme (Roche diagnostics, Mannheim, Germany), for 1-2 h at 4 ºC to equilibrate the sample to perform digestion. Digestion reaction was performed in a total volume of 200 µl containing 20 µl of H buffer, one third of plug (approximately 33 µl) and 9-20 U of *XbaI* enzyme, and was incubated overnight at 37 ºC. Finally, the reaction was interrupted by addition of 12.5 µl of EDTA 0.5 M pH 9-9.5, 12.5 µl of Sarcosyl 20 %, 25 µl of proteinase k (10 mg/ml) and incubating for 2 h at 50 ºC. Resulting DNA fragments were resolved by PFGE with the Gene Navigator equipment, the Power supply EPS60 (Pharmacia Biotech, Uppsala, Suede), and Contour-clamped Homogeneous Electric Field (CHEF) system (Chu *et al.*, 1986). To maintain the temperature of the buffer between 12 and 15 ºC, electrophoresis tray was connected to water refrigeration circuit (Heto Lab Equipment, Denmark). Samples and low range PFGE marker, which is a mixture of lambda DNA-Hind III fragments and lambda concatemers (New England Biolabs, Maine, USA), were charged in 1 % agarose gel in hepes buffer (annex I). The GN controller (Pharmacia Biotech, Uppsala, Suede) allowed to program a run with pulses of 5 to 25 s, for 22 h and with a voltage of 200 V and 120 mA. The electrophoresis gel was stained and viewed as describes 3.2.1. section (*Amplification of 16 S rDNA gene*).

3.2.3. Development of molecular markers

In this section the development and evaluation of specificity of two molecular markers designed for strain EPS125 by means of PCR and Real-time PCR methods was performed.

DNA extraction

Genomic DNA of strain EPS125 and bacterial strains listed in Table 3.4 was extracted following the methodology described by Keel *et al.* (1996). One isolated colony was transferred to 600 µl of LB broth and was grown for 24 h at 25 ºC. Then, 10 µl of overnight cultures were transferred to 90 µl of lysis buffer (annex I) and incubated at 99 ºC for 10 min. Finally, DNA extractions were stored at -20 ºC.

Table 3.4. List of bacterial strains used to determine the specificity of 125.2 and 125.3 molecular markers for strain EPS125.

Species	Code	Origin[a] or reference
Erwinia amylovora	EPS101	UdG
E. amylovora	1430	CFBP
Pantoea agglomerans	257 strains from fruit	UdG
P. agglomerans	850, 4842	CECT
Pseudomonas corrugata	124T	CECT
Pseudomonas fluorescens	EPS62e	UdG
P. fluorescens	324T, 845, 4064, 4518	CECT
Pseudomonas syringae pv. *syringae*	EPS40, EPS145	UdG

[a] UdG, Universitat de Girona (Spain); CFBP, Collection Française des Bactéries Phytopathogènes; CECT, Colección Española de Cultivos Tipo (Spain).

Search and design of DNA molecular markers

Two interesting genomic sequences of strain EPS125 obtained in this work (see annex II), which did not present any identity with known sequences deposited in GenBank data base, were selected to develop a DNA specific marker for strain EPS125.

Two primer sets (125.2f/125.2r and 125.3f/125.3r) and two TaqMan® probes (125.2s and 125.3s) were designed using Primer Express™ software (PE Applied Biosystems, MA, USA). TaqMan® probes were marked with a 6-carboxyfluoresceine (FAM) reporter dye (PE Applied Biosystems) at the 5' end, and both probes contained a 6-carboxy-tetramethyl-rhodamine (TAMRA) at the 3' end as the quencher. The sequences of primers and probes, as well as, the length of the amplified product are listed in Table 3.5.

The thermodynamic and interactions that occur among primers of both molecular markers and their respective probes were analysed *in silico* by using fold DNA bimolecular option from RNA structure 4.3 program (Mathews *et al.*, 2004).

Table 3.5. Primers and probes designed in this work to detect the strain EPS125.

Primers and probes	Sequence (5'→3')	Amplified product (bp)
125.2f	TGC GGG ACC CGA TTA TAT TG	
125.2r	GAT AGC GTT CGG CAC GGT TA	147 [(1)]
125.2s	CCA AGC AGG CAG CCT CTG TGG AC	
125.3f	CGG TAG GGC CAT CAT TCA TAA AT	
125.3r	TGG AAA CTA TTG ATG AAT TCT CAC CTT	135 [(2)]
125.3s	CCC GCT GTA GCA GCT CTC CAT GAA CAT AA	

r: reverse; f: forward; s:probe

[(1)] Design developed from sequence of 2126 mutant; [(2)] design developed from sequence of 40 mutant (annex II).

Specificity of DNA molecular markers by conventional PCR

Specificity of both primer pairs, 125.2 and 125.3, was tested against 257 strains of *E. herbicola* and 10 strains of different species (Table 2.4). A negative control without DNA and a positive control with EPS125 DNA were included in each reaction.

The amplification reactions were performed in a final volume of 50 µl containing 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each primer, 0.75 U of *Taq* DNA polymerase (Invitrogen) and 1 µl of the extracted DNA. The thermocycle conditions consisted of an initial denaturation step at 94 ºC for 2 min, followed by 35 cycles of denaturation at 94 ºC for 30 s, annealing at 60 ºC for 30 s, elongation at 72 ºC for 30 s and ending with an elongation step at 72 ºC for 7 min.

PCR products and 1 Kb plus ladder marker at 50 ng/µl (Invitrogen) were separated by horizontal electrophoresis and visualized as explains the 3.2.1 section (*Amplification of 16S rDNA gene*).

Specificity of DNA markers by Real-time PCR

Specificity of Real-time PCR was assessed with extracted DNA from 257 *E. herbicola* and 10 strains of other species (Table 2.4). A negative and a positive control were included in each PCR run and all reactions were performed in duplicate.

The concentrations of $MgCl_2$, primers and probe were optimised and the PCR reactions were carried in a final volume of 20 µl containing 1X PCR TaqMan Buffer A (PE Applied Biosystems), 6 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of each primer, 0.2 µM probe, 1 U AmpliTaq Gold® DNA Polymerase (PE Applied Biosystems) and 1 µl of the extracted DNA. The termocycle conditions consisted of an initial denaturation step at 95 ºC for 10 min followed by 50 cycles of 95 ºC for 15 s and 60 ºC for 1 min.

4. Results

4.1. Phenotypic characterization

4.1.1. Biochemical, morphological and complementary tests

Results obtained from biochemical, morphological and complementary tests performed on strain EPS125 are summarized in the Table 4.1.

Table 4.1 Morphological, biochemical and complementary characteristics of strain EPS125 in comparison with expected results for *Pantoea agglomerans* species (Brenner *et al.*, 1984).

Prove	**Result** [x]	***P. agglomerans*** [x]
Gram	negative	negative
Morphology	rod shaped	rod shaped
Pigmentation	yellow-orange	yellow-orange
Motility	+	+
Growth at 37 ºC	+	+
Glucose oxidation	+	+
Glucose fermentation	+	+
Voges-Proskauer	+	+ (v)
Oxidase	-	-
HR	-	- (v)
INA	-	- (v)
Production of IAA	-	v
Production of chitinases	-	v

([x]) **+**, positive; **–**, negative results; v, variable

Using API 20E strips the numeric profile 1005133 was obtained for the strain EPS125, corresponding to *Enterobacter agglomerans* (synonymous of *Pantoea agglomerans* and *Erwinia herbicola*) species with a 96.7 % of identity. Results of different metabolic tests are shown in Table 4.2.

Table 4.2. API 20E strip tests for the strain EPS125 in comparison with expected results for *Pantoea agglomerans* species (Brenner *et al.*, 1984).

Tests	**Result** [x]	**Tests**	**Result** [x]
β-galactosidase	+	glucose utilization	+
arginine dihydrolase	-	mannitol	+
lysine descarboxylase	-	inositol	-
ornithine descarboxylase	-	sorbitol	-
citrate utilization	-	rhamnose	+
H_2S production	-	sucrose	+
urease	-	melibiose	-
tryptophan deaminase	-	amygdalin	+
indole production	-	arabinose fermentation	+
acetoin production	+	oxidase	-
gelatinase	-		

([x]) **+,** positive; **–**, negative results

Strain EPS125 showed the antibiotic sensitivity spectrum detailed in Table 4.3.

Table 4.3. Sensitivity of the strain EPS125 to several antibiotics.

Antibiotic	Result (x)
ampicillin (100 µg/ml)	R
carbenicillin (100 µg/ml)	R
chloramphenicol (25 µg/ml)	S
spectinomycin (25 µg/ml)	R
streptomycin (10 µg/ml)	S
gentamicin (50 µg/ml)	S
kanamycin (100 µg/ml)	S
rifampicin (50 µg/ml)	R*
tetracycline (25 µg/ml)	S

(x) R, resistant; S, sensitive to antibiotic at tested concentrations. R* spontaneous mutant obtained by counterselection with rifampicin.

4.1.2. Utilization of carbon sources

Figure 4.1 shows the dendrogram obtained in relation with the utilization of 95 carbon sources by strain EPS125 in comparison with eleven strains pertaining to the same species and with two strains of *P. fluorescens*. Concretely, the cluster analysis defined two groups at rescaled distance of 25, one group formed by strains of *P. fluorescens* and another group was constituted of *E. herbicola* strains, which included strain EPS125.

The largest difference between the two clusters was due to the utilization of the following carbon sources: maltose, β-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketobutyric acid, L-histidine, L-leucine, L-pyroglutamic acid, L-threonine, D,L-carnitine, putrescine and 2-

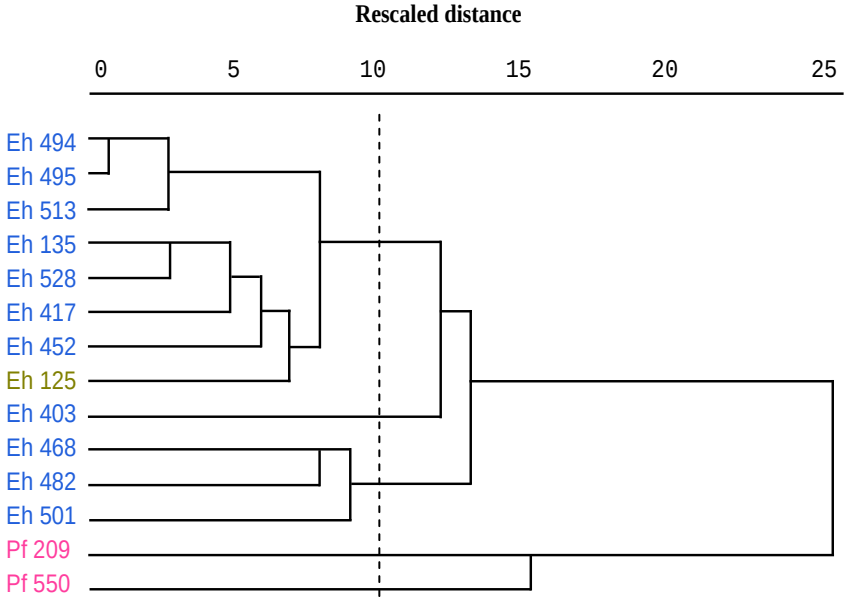

Figure 4.1. Dendrogram indicating the relatedness between the strain EPS125 and eleven strains pertaining to the same species and two strains of *P. fluorescens* according to Biolog GN data. Euclidean distances were calculated on the basis of the similitude and differences between profiles. These data were clustered using the agglomerative hierarchical method. Dashed line represents the threshold used for grouping.

aminoethanol. In addition, three clusters were observed into the *Pantoea* group at rescaled distance of 10, where strain EPS125 was included in cluster 1. This strain was unable to use tween 40 at difference of strains from the same cluster and to metabolize mono-methyl succynate at difference of all analyzed strains.

4.1.3. Fatty Acid Methyl Ester (FAME) profile

Fatty acids considered to perform the analysis were the 10:0,10:0 ISO, 12:0, 14:0, 15:0, 16:0, 17:0, 18:0 of saturated group; 18:1 w7c, 18:1 w5c, ISO 17:1 w5c of unsaturated group; 10:0 3OH, 12:0 2OH, 12:0 3OH, 14:0 2OH and 17:O ISO 3OH of hydroxy group; 17:0 cyclo, 19:0 cyclo w8c of cyclic group and the unknown (unk) fatty acids 14.502, 18.814 and 13957 (Table 4.4).

Table 4.4. Fatty acid relative content of several strains analyzed in this work in comparison to EPS125

	Strain													
Fatty acid	Eh501	Eh468	Eh482	Eh452	Eh494	Eh417	Eh528	Eh495	Eh403	Eh513	Eh125	Eh850	Eh4842	Pf550
10:O	-	-	-	0,50	-	-	-	-	-	-	-	-	-	-
12:O	2,49	2,58	3,19	2,57	2,37	2,52	2,70	2,23	3,21	2,82	3,70	2,35	2,15	2,04
14:O	4,21	5,13	4,45	5,06	4,56	4,30	4,83	4,97	1,88	4,40	4,75	5,33	6,61	0,55
15:O	0,54	-	0,54	-	-	-	0,57	0,60	0,98	-	0,76	-	0,59	-
16:O	26,05	28,12	27,06	26,87	26,59	25,97	26,55	24,66	30,77	26,11	25,04	30,53	30,72	24,33
17:O	0,62	0,59	0,60	0,77	-	0,61	0,85	-	1,35	0,52	0,54	0,60	0,63	-
14:0 2OH	-	-	-	-	-	-	-	-	1,99	-	-	-	-	-
17:0 anteiso	0,73	-	0,56	-	-	-	-	1,32	-	-	-	-	-	-
17:0 cyclo	5,05	5,04	3,47	5,75	7,66	6,60	6,28	6,53	5,40	5,36	10,29	6,34	7,91	2,30
18:1w7c	17,35	19,41	19,04	18,59	18,89	17,73	19,16	18,10	15,11	17,49	15,48	15,97	25,10	22,74
18:1w5c	-	0,56	-	-	-	-	-	-	-	-	-	-	-	-
SIF 2	7,52	5,40	6,38	6,77	6,68	8,05	7,52	7,30	7,02	8,69	7,01	6,48	5,07	-
SIF 3	28,06	30,45	29,34	26,16	27,84	28,29	26,31	24,98	23,82	29,11	25,43	30,69	19,27	32,27
SIF5	1,23	-	0,70	-	-	-	-	2,07	1,38	-	0,52	-	-	-
unk 14,502	1,42	0,82	1,37	1,51	1,22	1,12	1,27	1,54	1,44	1,38	-	0,84	0,86	-
unk 18,814	0,97	-	-	0,51	-	-	-	1,30	0,61	-	-	-	-	-
10:0 ISO	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10:0 3OH	-	-	-	-	-	-	-	-	-	-	-	-	-	3,87
12:0 2OH	-	-	-	-	-	-	-	-	-	-	-	-	-	4,64
12:0 3OH	-	-	-	-	-	-	-	-	-	-	-	-	-	3,85
18:O	0,60	0,53	0,59	0,70	0,5	0,53	0,70	0,76	0,64	0,53	-	0,89	0,61	1,41
unk 13957	0,73	-	0,71	-	0,85	-	-	-	0,80	0,75	0,69	-	-	-
17:O iso 3OH	0,65	-	-	-	-	-	-	0,67	-	-	0,59	-	-	-
iso 17:1 w5c	-	-	-	-	-	-	-	-	0,53	-	-	-	-	-
19:O cyclow8c	-	-	-	-	-	-	-	-	0,67	-	-	-	-	-
14:1 w5c	-	-	-	-	-	-	-	-	-	-	0,67	-	-	-
SIF1	-	-	-	-	-	-	-	-	-	-	0,63	-	-	-
15:O iso 3OH	-	-	-	-	-	-	-	-	-	-	0,74	-	-	-

(-) Lower values than 0.5 of fatty acid relative content.
(SIF) Sum In Feature. SIF2 value is the addition of 14:0 3OH, 16:1 ISO I and unk 10.928 peaks. SIF3 value is the addition of 15:0 ISO 2OH and 16:1w7c peaks. SIF5 value is the addition of 18:0 ANTE and 18:2 w6, 9c peaks.

From FAME profile of each analysed strain, the dendrogram showed in the Figure 4.2 was obtained. At rescaled distance of 25, two groups were defined according to their fatty acids composition and relative proportion, one group made up of *P. fluorescens* strains and another formed of *E. herbicola* strains including the strain EPS125. No defined cluster was showed below rescaled distance of 20 for *E. herbicola-P. agglomerans*, which formed a progressive gradient.

P. fluorescens group is different from *E. herbicola* group due to its low relative contain of 14:0 and 17:0 cyclo fatty acids, the high SIF3 value, the presence of 10:0 3OH, 12:0 2OH and 12:0 3OH groups and the absence of SIF2 value.

Strain EPS125 contains the majority of fatty acids and in the same relative content described in strains of *E. herbicola* species. However, in comparison with the other *E. herbicola* strains, it has a larger content of 17:0 cyclo fatty acid and a low content of 14:1 w5c, SIF1, 15:0 ISO 3OH, and a less relative content of 18:0 than 0.5.

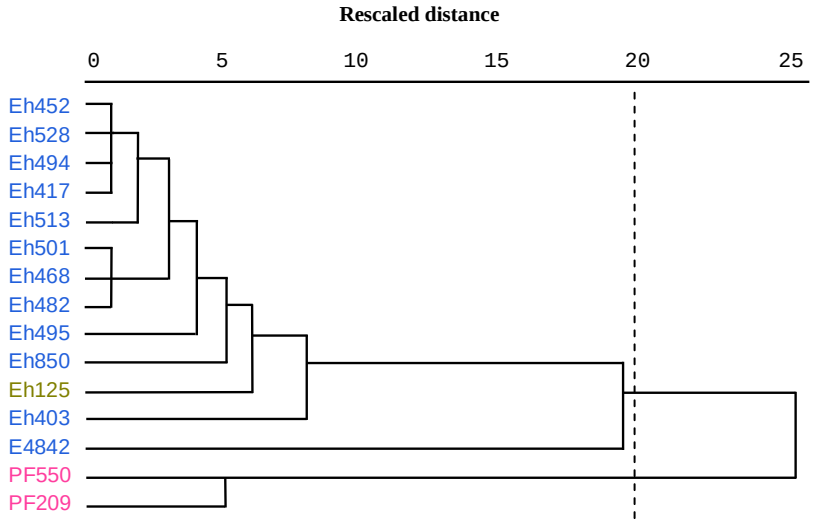

Figure 4.2. Dendrogram indicating the relatedness between the strain EPS125 (indicated as Eh125) and 12 strains pertaining to the same species and 2 *P. fluorescens* by means of FAME profiles. Euclidean distances were calculated according to the differences of composition and relative content of several fatty acids between strains. These data were clustered using the agglomerative hierarchical method. Dashed line represents the threshold used for grouping.

Table 4.5. Identification of strains according to the relative content of fatty acids of membrane by means of Sherlock program.

Strain	Identification	Identity values
Eh-EPS125	*Enterobacter-E. agglomerans* (*Pantoea*)	0.6-0.4
Eh-EPS 403	*Pantoea* (*Erwinia ananas)*	0,7
Eh-EPS 417	*Enterobacter*	0,7
Eh-EPS 452	*Erwinia*	0,6
Eh-EPS 468	*Erwinia*	0,6
Eh-EPS 482	*Erwinia*	0,6
Eh-EPS 494	*Enterobacter*	0,6
Eh-EPS 495	*Enterobacter*	0,4
Eh-EPS 501	*Enterobacter*	0,6
Eh-EPS 513	*Enterobacter*	0,7
Eh-EPS 528	*Enterobacter*	0,6
CECT850	*Pantoea* (*agglomerans*)	0,8
CECT4842	*Enterobacter-E. agglomerans* (*Pantoea*)	0.8-0.6
Pf-EPS 209	*Pseudomonas* (*syringae*)	0,6
Pf-EPS 550	*Pseudomonas* (*putida*)	0,7

FAME profiles were processed by Sherlock program to obtain identity values of analyzed strains having as reference strains deposited in the MIS Microbial Identification System database. Equal or higher identity values than 0.6 were considered as correct identification. The EPS550 and

EPS209 strains pertained to the first group of above dendrogram (*P. fluorescens* group) were identified as *Pseudomonas* spp. (*P. putida* and *P. syryngae* respectively). The majority of strains pertained to the second group (*E. herbicola*) were identified as *Erwinia* spp., *Pantoea* spp. and *Enterobacter* spp. (*E. agglomerans*) with identity values about 0.6. Specifically, the strain EPS125 was identified as *Enterobacter* spp. with a identity value of 0.6 and as *E. agglomerans* (*Pantoea* spp.) with a identity value of 0.4 (Table 4.5).

4.1.4. Dose-response assays

The effect on disease levels of the application dose of *P. agglomerans* EPS125 and the *P. expansum* on apple is shown in Figure 4.3 as disease levels. From these severity results, efficiency parameters presented in Table 4.6 were estimated using the hyperbolic saturation (HS) model. It was observed that the data fitted well to the HS model because values of MSE ranged from 0.0045 to 0.0190 and the asymptomatic standard errors (ASE) were lower than parameter values (Y_{max} and I_{max}). The Y_{max} (maximum disease suppression) and I_{max} (maximum proportion of pathogen inactivated) values were approached 1 indicating that all fruit were susceptible to disease and that all pathogen was inactivated by strain EPS125, respectively. The values of K_z (ED_{50} biocontrol agent) compared to K_x (ED_{50} pathogen) were less variable and ranged from $2.69x10^5$ and $7.02x10^5$ cfu/ml. The ratio K_z/K_x results obtained, of 101 for the first assay and 25 for the second, show that EPS125 was more effective in the second assay as it was needed 25 cfu of BCA per pathogen spore to get a 50 % biocontrol. High concentrations of EPS125 (10^8, 10^9 cfu/ml) produced a full inhibition of disease production by *P. expansum* at $5x10^2$ and 10^3 spores/ml concentrations, but a 15 and 20 % of residual infections were not controlled at 10^4 spores/ml concentration.

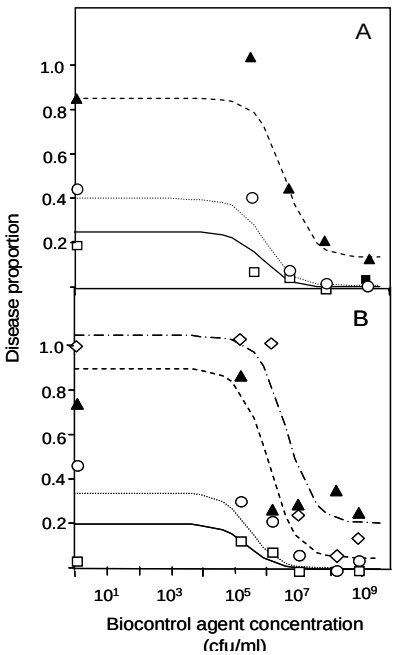

Figure 4.3. Infectivity titration of *Penicillium expansum* on Golden apple fruits wounded and treated with increasing concentrations of *P. agglomerans* EPS125. The pathogen densities were 5 x 10^2 (□), 1 x 10^3 (○), 1 x 10^4 (▲), and 5 x 10^4 (◊) conidia/ml. The lines represent predictions of disease proportion at the different pathogen concentrations according to a hyperbolic saturation model, using estimated parameters shown in Table 4.2

Table 4.6. Estimated parameters using hyperbolic saturation model that relates disease severity with different pathogen and biocontrol agent concentrations.

Assay	DFE[a]	Parameter				MSE
		Y_{max} (maximum disease proportion)	K_x (ED_{50} pathogen)[b]	I_{max} (maximum pathogen proportion inactivated)	K_z (ED_{50} biocontrol agent)	
1	10	1.15 (0.10)	$1.39x10^3$ ($3.4x10^2$)	0.97 (0.009)	$7.02x10^5$($1.37x10^5$)	0.0045
2	20	1.08 (0.09)	$2.17x10^3$ ($9.8x10^2$)	0.99(0.005)	$2.69x10^5$ ($1.27x10^5$)	0.0190

[a] DFE, degrees of freedom for the error; MSE, mean square error. The assymptotic standard errors (ASE) for the parameter estimates are given in parentheses.
[b] Densities for pathogens are in conidia/ml and for *P. agglomerans* EPS125 are in cfu/ml.

4.2. Genotypic characterization

4.2.1. Sequencing of 16S rDNA

From DNA of strains EPS125, CECT850T and CECT4842, one amplified fragment of approximately 1500 bp of 16S rDNA gene obtained with 8f and 1492r primers was sequenced (Figure 4.4). Once DNA was extracted from gel, it was proceeded to their total sequencing with primers described in the Table 2.2. Partial sequences of 16S rDNA gene of the three strains were deposited in GenBank database with the codes AJ583011 (EPS125), AJ583835 (CECT850T) and AJ583836 (CECT4842).

The sequences of the 16S rDNA gene of strains EPS125, CECT850T and CECT4842 were analysed with BLAST option (http://www.ncbi.nlm.nih.gov/BLAST) to compare with sequences deposited in the GenBank database. The sequence of strain EPS125 was closely related with sequences of *Enterobacter*

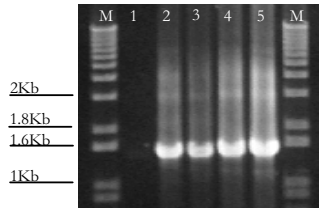

Figure 4.4. Amplified products of 16S rDNA gene (~1.6 Kb). (1) negative control (without DNA), (2,5) EPS125, (3) CECT850T, (4) CECT4842, (M) 1Kb plus DNA ladder (Invitrogen).

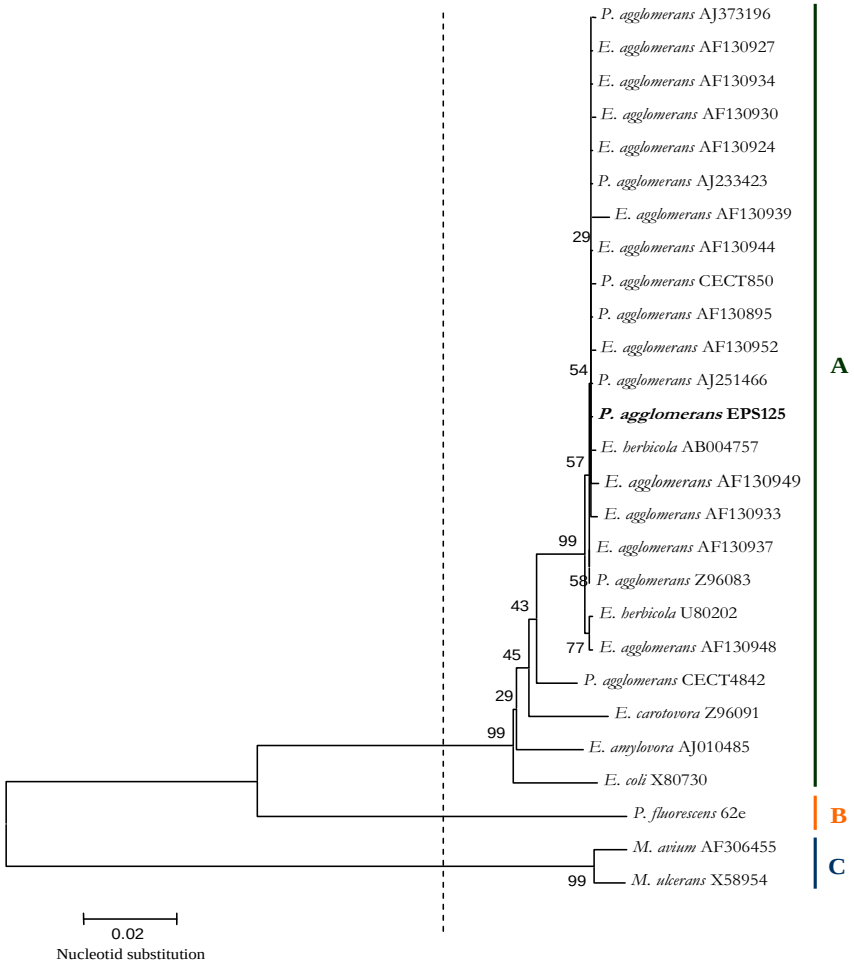

Figure 4.5. Phylogenetic tree showing interrelationships among 16S rDNA sequences of strains EPS125, CECT850T and CECT4842 and other deposited in GenBank database. Numbers labelled above each node are bootstrap values. Scale shows the substitution of nucleotides for each position on sequence (2 %). Dashed line represents the threshold used for grouping.

and *Pantoea* genera, showing a 99 % of identity with strains *P. agglomerans* LMG2 (1390 of 1391 bases) and new*45con (1378 of 1391 bases). The sequence of strain CECT850T of 1473 bases had a 99 % of identity with sequences of *P. agglomerans* species. However, the sequence of 1452 bases of strain CECT4842 had a 98 % of identity with sequences of *Klebsiella oxytoca* species. In addition, these sequences were compared with other sequences of 16S rDNA gene of close and distant phylogenetic bacteria. The phylogenetic tree (Figure 4.5) shows three groups; the first group (group A) is made up of *P. agglomerans*, *E. agglomerans*, *E. herbicola*, *Erwinia carotovora*, *E. amylovora* and *Escherichia coli* enteric bacteria; the second group (group B) is constituted of the only specie *Pseudomonas fluorescens*, and the third group (group C) comprises two strains of *Mycobacterium* genus. Strain EPS125 is included in the group A, being more closed to strains of *P. agglomerans*, *E. agglomerans*, *E. herbicola* species than to strains of *E. carotovora*, *E. amylovora* and *E. coli*.

4.2.2. Macrorestriction Fragment Length Polymorphism analysis (MRFLP)

As Figure 4.6 shows, strain EPS125 has a characteristic macrorestriction fragment length polymorphism upon digestion of DNA with *XbaI* consisting of 13 fragments of 302, 270, 259, 253, 197, 179, 165, 160, 140, 110, 100, 81 and 71 Kb (Table 4.6). This electrophoretic pattern differs from other strains of *E. herbicola* species such as EPS131, EPS512, EPS454 and EPS549. In addition, this polymorphism was maintained in several reisolates of strain EPS125 from apples that were treated with the antagonistic strain in previous postharvest assays (Figure 4.6).

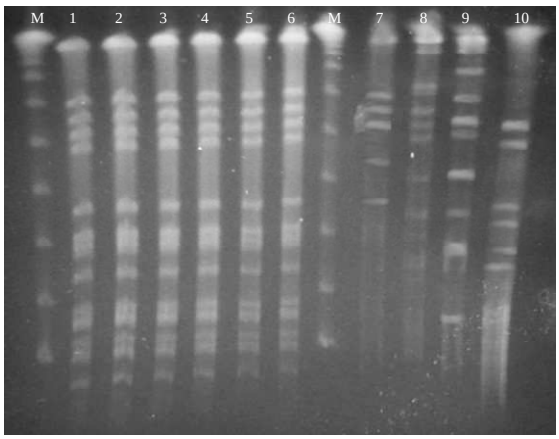

Figure 4.6 Genomic macrorestriction patterns of strain EPS125 and several *E. herbicola* strains after *Xba* I digestion. (1) EPS125, (2-6) EPS125 reisolates from apple, (7) EPS131, (8) EPS512, (9) EPS454, (10) EPS549, (M) Low range PFGE marker (NEB).

Table 4.6. Number and length of fragments produced by digestion of genomic DNA with *Xba* I restriction enzyme. PFGE run conditions solved DNA fragments from 50 to 315 Kb of length with a variation about 10 Kb.

Strain	Number of fragments	Length (Kb)
EPS125	13	302, 270, 259, 253, 197, 174, 165, 160, 140, 110, 100, 81, 71
EPS131	5	283, 264, 247, 231, 194
EPS454	7	274, 260, 252, 215, 180, 151, 86
EPS512	6	315, 260, 257, 250, 213, 180
EPS549	9	264, 245, 184, 169, 147, 133, 98, 84, 61

4.2.3. Development of molecular markers

The molecular markers described in this section to detect *P. agglomerans* EPS125 (125.2 and 125.3) were developed from two DNA sequences of this strain that did not present similarity with any nucleotide and aminoacidic sequence deposited in the GenBank database (details in Chapter II and annex II).

The specificity of these two molecular markers (125.2 and 125.3) was tested against a total of 267 bacteria, 257 strains of *P. agglomerans (E. herbicola-E. agglomerans*) species and 10 strains of related species. The 125.2 primer pair was semispecific for the amplification of genomic DNA from EPS125, showing a single amplification product with the expected length of 147 bp in 5 *E. herbicola* strains (EPS426, EPS458, EPS467, EPS468 and EPS483). The 125.3 primer pair amplified, apart from EPS125, a fragment of 135 bp in 1 *E. herbicola* strain (EhEPS466), which is different from those detected with 125.2 primer set (Figure 4.7). In spite of both designs were semispecific for strain EPS125, their combined use achieved a specific detection of this strain. According to these results a multiplex PCR can be performed for specific detection and quantification of strain EPS125.

Multiplex PCR is a variant of PCR which allows simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers (Higuchi *et al.*, 1992). The thermodynamic and interactions of both primer sets and respective probes are detailed in the Figure 4.9, and all combinations showed optimal conditions with low levels of interaction energy (ΔG or E), which is essential to perform multiplex PCR.

The specificity of the 125.3 design was tested by means of Real-time PCR. A successful amplification of strain EPS125 was achieved with a C_T value about 18.5 and a threshold of 0.02 (Figure 4.8). However, a positive signal was observed in one *E. herbicola* strain (EhEPS466), which was the same as was amplified by qualitative PCR. The rest of the analyzed strains showed a background fluorescence signal with C_T values >31, which were considered as a negative result. Nonetheless, a multiplex PCR using both primer sets have not been developed yet.

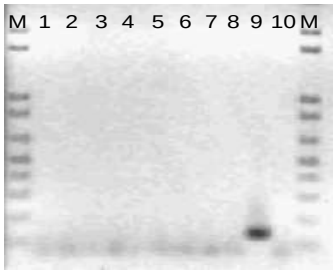

Figure 4.7. Screening of the 125.3 primer specificity. (1) Eh-EPS205, (2) Eh-EPS281, (3) Eh-EPS343, (4) Eh-EPS478, (5) Eh-EPS622, (6) Eh-EPS625, (7) Ea-EPS101, (8) Ea-EPS1430, (9) Eh-EPS125, (10) negative control without DNA, (M) 1 Kb Plus DNA Marker.

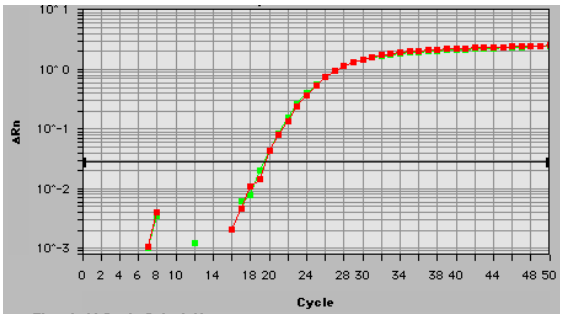

Figure 4.8. Real-Time PCR detection and amplification of *P. agglomerans* EPS125 by using 125.3 molecular marker.

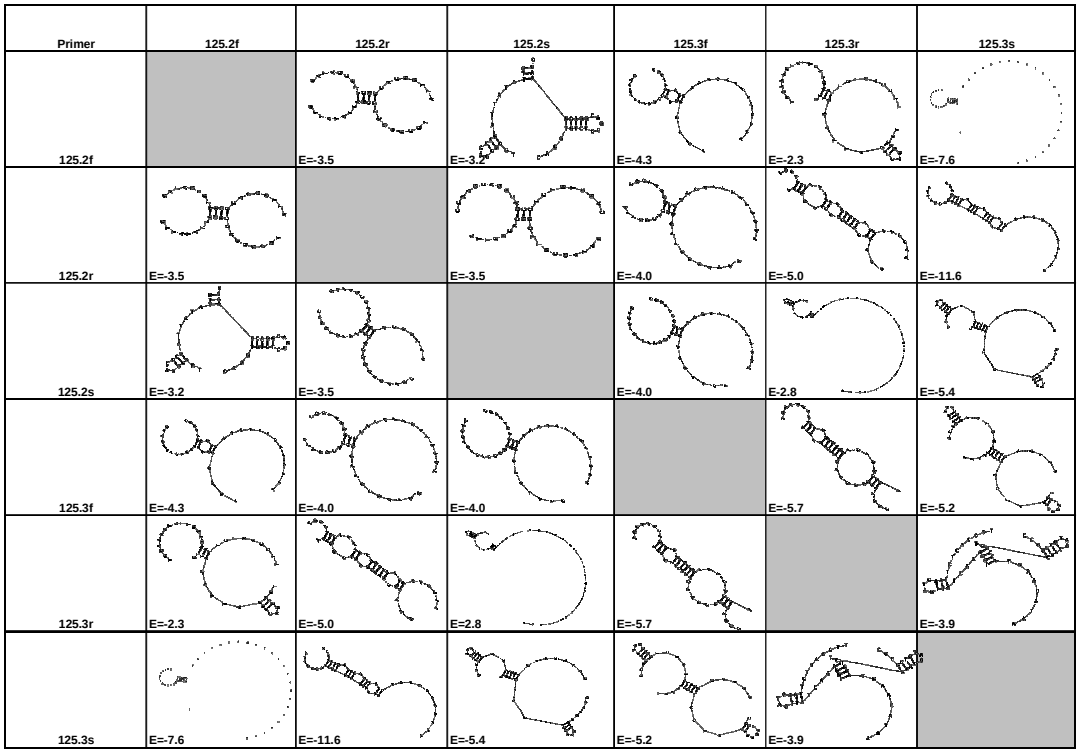

Figure 4.9. Scheme showing the kind of molecular interaction and the interaction energy between primers and probes of 125.2 and 125.3 molecular markers by means of the RNA structure 4.3 program (Mathews *et al.*, 2004).

5. Discussion

Strain EPS125 was isolated from a pear fruit surface being an effective biocontrol agent against blue mould of apple and pear caused by *Penicillium expansum* (Vázquez, 1998). However, it has not been characterized at phenotypic and genotypic levels yet. For this purpose, a polyphasic characterization of strain EPS125 was performed in this chapter.

All results obtained from classic morphological and biochemical tests classified strain EPS125 into *Pantoea agglomerans* species according to Brenner *et al.* (1984). In addition, this strain showed 96.7 % of identity with *Enterobacter agglomerans* species using API 20E strips. Nonetheless, strain EPS125 did not use citrate as the only external carbon source as it has been described in other *P. agglomerans* strains.

FAME profile is a powerful tool to discriminate strains between genera and sometimes between bacterial species (Sasser, 1990), as well as to characterize strains from different species (De Baere *et al.*, 2002) or under the same species (El Hendawy and Azab, 1999; Ellis *et al.*, 2000). Values of fatty acid relative contents of EPS125 included this strain into the genus *Enterobacter* with a 0.6 value of similarity and into the species *E. agglomerans* (*Pantoea*) with a value of 0.4. In most cases, Sherlock program has identified strains of species *E. herbicola* as *Enterobacter*, *E. agglomerans*, *Erwinia* and *Pantoea*, and strains of species *P. fluorescens* as *Pseudomonas* (*Pseudomonas putida* and *Pseudomonas syringae*) with a similarity value equal or greater than 0.6. However, it has to take into account these results are submitted to limitation of FAME taxonomic library that is mostly performed by FAME profiles from pathogenic bacteria, concretely human and plant pathogens. Several works have showed the limitations of taxonomic library to identify strains using fatty acid profiles. For example, strains identified as *P. fluorescens* Q 2-87, CHAO, 2-79 and F-113 in the bibliography were identified as *Pseudomonas aureofaciens*, *Pseudomonas putida* bv. a, *Pseudomonas marginalis* and *Pseudomonas savastanoi*, respectively (Harrison *et al.*, 1993; Ellis *et al.*, 2000). In addition, the same FAME values allowed the construction of a dendrogram according to fatty acid profiles of all analyzed strains. This dendrogram showed 2 groups composed of *E. herbicola-E. agglomerans-P. agglomerans* and *P. fluorescens* strains, being strain EPS125 included in the first group. FAME analysis not only allows to identify and characterize strains, but also to perform other interesting studies such as the work performed by Ellis *et al.* (2000). They have found that C17:0 (17CFA) fatty acid accumulation and cyanhydric acid production of several strains pertaining to *Pseudomonas fluorescens* were significantly correlated with their biocontrol activity against several phytopathogen fungi. The 17CFA was proposed as phenotypic marker to select new *P. fluorescens* biocontrol agents. Although, we did not perform FAME characterization of the strain EPS125 to find a biocontrol marker, nonetheless a noteworthy 17:0 CYCLO relative contents was observed being greater than in other strains from the same species.

The Biolog method is another identification system based on the comparison of oxidation profiles of several carbon sources with profiles of microorganisms that were introduced and identified in the database. This system also presents the disadvantage of using taxonomic libraries as FAME and, moreover, it is very influenced by inoculum density, which is less important in the identification by means of API 20E method (Makemson *et al.*, 1998). Biolog plates have been used in many works to classify and characterize several bacterial species (Makemson *et al.*, 1998; Verma *et al.*, 2001; Ashelford *et al.*, 2002) and to perform intra-specific studies comparing strains. For instance, in a work performed by Gardener *et al.* (2000), Biolog plates were used to determinate the diversity between several strains of *Pseudomonas fluorescens* that produced phloroglucinol. They found that the main difference between strains felt on proportion that each carbon source was oxidized and not on the number or the kind of carbon source. In the present work, Biolog plates were used to characterize strain EPS125 in comparison with other strains pertaining to *E. herbicola-P. agglomerans* and *P. fluorescens* groups according to their profiles of carbon source utilization. The dendrogram obtained from these

data showed two groups corresponding to *Pantoea* and *Pseudomonas* species clearly differed. Strain EPS125 was included in the *E. herbicola-P. agglomerans* reasserting the identification of strain EPS125 as *P. agglomerans*. In addition, as much FAME as Biolog methods have shown several traits of strain EPS125 that makes it different from strains of the same and different species. For example, strain EPS125 has fatty acids 14:1 w5c, SIF1 and 15:0 ISO 3OH, as well as it is unable to use tween 40 at difference of strains from the same cluster and to metabolize mono-methyl succynate at difference of all analyzed strains. Therefore, these results show that the biocontrol agent EPS125 has singular characteristics different from other *E. herbicola-P. agglomerans*.

In the development of a commercial biopesticide, neither pathogenicity nor ice nucleation activity (INA) have to be present, as these are properties harmful to plant crops. Several works have demonstrated that in general species *E. herbicola* are not plant pathogens (Dye, 1969; Ewing and Fife, 1972; Gavini *et al.*, 1989). Only two pathovars from this species, pv. *gysophilae* and pv. *betae*, are pathogens of *Gypsophila paniculata* and beetroot, respectively (Cooksey, 1986). In this work the absence of hypersensitivity response in tobacco plants of strain EPS125 has been demonstrated. Several strains pertaining to *P. fluorescens* and *E. herbicola* species are INA positive and they may produce frost damage on plants. However, the biocontrol agent EPS125 did not show ice nucleation activity.

The antagonist-pathogen density relationships obtained from dose-response assays provides data on the population levels of antagonist required to achieve adequate disease control (Johnson, 1994) as well as provide efficiency parameters of the antagonist strain allowing the comparison with other pathosystems (Johnson, 1994; Raaijmakers *et al.*, 1995; Montesinos *et al.*, 1996; Smith *et al.*, 1997; Larkin and Fravel, 1999; Francés *et al.*, 2006). The ratio between the median effective dose (ED_{50}) of the biocontrol agent and the pathogen in the hyperbolic saturation model (*Kz/Kx*) is one of the most useful parameters and this allows to measure the efficiency of the biocontrol agent in terms of cells of the BCA needed to inhibit a pathogen cell (Montesinos *et al.*, 1996). According to the results stated in the present work, strain EPS125 was highly effective against *P. expansum* in apple fruit showing ED_{50} from $2.7x10^5$ to $7x10^5$ cfu/ml (Francés *et al.*, 2006). These values agreed with the ED_{50} reported by Bonaterra *et al.* (2003) and Francés *et al.* (2006) for strain EPS125 in other pathosystems. In the first work, strain EPS125 showed an ED_{50} of $0.5x10^5$ and of $2x10^5$ cfu/ml when it was treated on stone fruit together with *Monilinia laxa* and *Rhizopus stolonifer*, respectively. And in the second study, strain EPS125 showed an ED_{50} from $1.5x10^5$ to $1.6x10^5$ CFU/ml when it was treated on strawberry cv. 'Pájaro' with *Botrytis cinerea*. However, strain EPS125 was more efficient in controlling *P. expansum* in apple fruit than in other pathosystems; because 25-101 EPS125 cells were needed to inactivate one conidium of *P. expansum*. On the contrary, 166 EPS125 cells were needed to inhibit one conidium of *M. laxa*; 1263 EPS125 cells were needed to inactivate one sporangiospore of *R. stolonifer*; and $4.3x10^4$ cells were needed to inhibit one pathogen cell of *B. cinerea*. These values are similar to the median effective dose ratio reported in non-postharvest systems from 0.2 to $5x10^4$ propagules of fungus mycoparasite/propagule of fungal pathogen in soil pathosystems (Adams, 1990), 176 and 235 cfu/conidia in *P. fluorescens* and *P. agglomerans* against *S. vesicarium* on pear (Montesinos *et al.*, 1996) and from 245 to 7142 cfu/zoospore in *Bacilus cereus* UW85 against *Pythium torulosum* in several tomato cultivars (Smith *et al.*, 1997). Hence, EPS125 has shown to be an effective BCA with optimal action range at moderately concentrations in preventive treatments for the control of postharvest rots on several fruit cultivars.

Finally, strain EPS125 is naturally resistant to ampicillin, carbenicillin and spectinomycin, and susceptible to chloramphenicol, streptomycin, gentamicin, kanamycin and tetracycline. These results were decisive at the moment to choose an appropriate system of mutagenesis with transposons. Usually, resistances showed by recipient strain must not coincide with resistances conferred by transposon and vector used during mutagenesis as, in this way, it is

possible to ensure that true mutants have been obtained according to antibiotic resistance markers. However, whether to find an appropriate marker was not possible, other confirmation systems can be used. In addition, a spontaneous mutant resistant of EPS125 to rifampicin (50μg/ml) was selected and used for several studies (Bonaterra *et al.*, 2003; Francés *et al.*, 2006). This acquired resistance is related with a mutation in the gene coded for RNA polymerase subunit-β, the antibiotic target. In this way, rifampicin cannot bind to RNA polymerase subunit-β and the transcription process is not altered (Alvarado-Esquivel *et al.*, 2001).

Identification of naturally occurring gram negative bacteria according to phenotypic characteristics is a time consuming process and may be submitted to variability because of the influence of the environment. Moreover, methods used for identification of enterobacteria are generally imprecise (Toth *et al.*, 2001). Sequencing of 16S rDNA gene is a good method to identify microorganisms so it is not influenced by the gene expression variability such as phenotypic identification (Drancourt *et al.*, 2000). Several works have suggested the study of 16S rDNA sequences to identify the taxonomic relationships between several species and *Erwinia* group (Kwon *et al.*, 1997; Hauben *et al.*, 1998; Mergaert *et al.*, 1999; Kim *et al.*, 1999).

In the present work, 16S rDNA gene of EPS125, *P. agglomerans* CECT850T and *P. agglomerans* CECT4842 strains was sequenced with the purpose to identify and characterize strain EPS125. Sequences obtained of 1451 bp for the strain EPS125 and 1473 bp for the strain *P. agglomerans* CECT850T showed a 99 % identity with sequences deposited in Genbank database pertaining to *P. agglomerans* strains. However, the sequence of the strain CECT4842 of 1452 bp obtained a 98 % of identity with sequences from *Klebsiella oxytoca* strains. This last result is surprising as the strain CECT4842 is deposited and identified as *P. agglomerans* in the CECT collection. However, it explains its position in the dendrogram obtained according to FAME analysis, where the strain CECT4842 included in *E. herbicola – P. agglomerans* group was the nearest to the *P. fluorescens* group. Also, it fits well with the observation that strain CECT4842 is located in the phylogenetic tree constructed according to 16S rDNA sequences being the strain bordering *E. herbicola-P. agglomerans* cluster and other enterobacteria. The 16S rDNA dendrogram obtained in this work showed three groups made up of *Mycobacterium* genus, one *P. fluorescens* strain and enterobacteria group. Strain EPS125 was included in the third group being nearer to *E. agglomerans*, *E. herbicola* and *P. agglomerans* strains than *E. carotovora*, *E. amylovora* and *E. coli* strains. This result is just like the phylogenetic relationships obtained by Kwon *et al.* (1997) between strains of different species from the *Erwinia* genus according to 16S rDNA gene. Their results showed a high heterogeneity between species from the *Erwinia* genus, which was divided into four groups very related to other genera such as *E. coli*, *Klebsiella pneumoniae* and *Serratia marcescens*.

The genomic characterization based on macrorestriction fragment analysis separated by PFGE is a useful tool to perform intra-species studies, as it has a high power of discrimination until strain level (Tynkkynen *et al.*, 1999; Garaizar *et al.*, 2000; McLellan *et al.*, 2001; Badosa, 2003). PFGE technique has a larger discrimination power and more reproducibility of results than RAPD technique (Badosa, 2003; Garaizar *et al.*, 2000). For these reasons, pulsed field technique was chosen to perform the genomic characterization of strain EPS125. The electrophoretic pattern of strain EPS125 obtained by digestion of genomic DNA with *Xba I* endonuclease was composed of 13 characteristic bands allowing to differentiate it from other strains pertaining to the same species. So that, this specific pattern might be used as a monitoring method to describe and identify strain EPS125.

The MRFLP pattern of strain EPS125 allows to compare and distinguish it from other strains pertaining to the same species. However, MRFLP technique only can be performed from pure culture, thus requiring a previous step of isolation from samples. The development of DNA molecular markers for strain EPS125 is a monitoring tool simpler than MRFLP method. It is based on the presence or absence of a unique amplified fragment of known size from a DNA mixture. There are several works describing molecular markers specific for genus of *Erwinia*,

mycobacteria, and pseudomonads (Heuser and Zimmer, 2003; Khan and Yadav, 2004) and for strains of BCAs like *Candida oleophila*, *Paecilomyces lilacinus*, *Plectosphaerella cucumerina*, *Pichia anomala* and *Trichoderma harzianum* (Atkins *et al.*, 2003; Massart *et al.*, 2005; Rubio *et al.*, 2005; Atkins *et al.*, 2005; Pujol *et al.*, 2005). For example, Pujol *et al.* (2006) have developed a specific Real-time PCR for the detection and quantification of *P. fluorescens* 62e, an effective biocontrol agent against *Erwinia amylovora*. This method allowed to quantify the biocontrol agent under different conditions consisting of active colonisation of apple flowers and epiphytic phyllosphere survival in the greenhouse and field.

From genomic sequences of strain EPS125 that did not present homology with sequences deposited in the Genbank database, two molecular markers were designed (125.2 and 125.3). Each design consisted of a primer pair (125.2f/125.2r and 125.3f/125.3r) and a TaqMan® probe (125.2s and 125.3s). Firstly, an intra-specific and inter-specific evaluation of both molecular markers was performed by conventional PCR according to the presence/absence of an only amplified fragment of 147 bp for 125.2 design and 135 bp for 125.3 design. Both designs were semispecific as 125.2 design amplified an unspecific fragment on 5 *E. herbicola* strains and 125.3 primer pair 1 *E. herbicola* strain, from 257 *E. herbicola* strains. Specificity of the 125.3 design was tested by means of Real-time PCR and the same strain amplified above by PCR showed positive signal. Massart *et al.* (2005) found a similar result, they have described a sequence characterized amplified region (SCAR) of the postharvest biocontrol agent *Candida oleophila* O. The amplification product of 244 bp was semi-specific detecting three other *C. oleophila* strains. SCAR fragment of four microorganisms were sequenced to find polymorphisms between them and to develop a real-time PCR for specific detection of the biocontrol agent. A 3'-Minor Groove Binding (MGB) probe was designed to specifically match a region of the strain O sequence. This was able to discriminate a two-base difference in the corresponding sequences of the non-target strains. In this way, the real-time PCR method using a MGB probe allowed specific detection of strain O.

Owing to strain EPS125 was the only one that achieved amplification signal with both primer sets, a multiplex PCR could be developed allowing a simultaneous amplification of the two molecular markers in one reaction for its specific monitoring.

In conclusion, results obtained using phenotypic and genotypic methods shows that strain EPS125 pertains to *Pantoea agglomerans* species although it has several specific phenotypic and genotypic traits compared to other *P. agglomerans* strains. Therefore, this biopesticide has been deposited into the "Colección Española de Cultivos Tipo" according to established conditions of the Budapest agreement for patenting microorganisms as *Pantoea agglomerans* CECT5392, and a patent has been sought for its application as biopesticide of fungal moulds in postharvest covering several European countries.

Chapter II
Mechanism of action of *Pantoea agglomerans* EPS125

1. Introduction

To reach enough consistency of control from trial to trial with the application of a biopesticide, it is necessary to fully understand interactions that occur between the biological control agent, the pathogen, the host plant, other surrounding microorganisms and the environment. This knowledge allows to develop strategies to improve conditions of interaction between microorganisms increasing biocontrol efficacy, which also depends on intrinsic aggressiveness of the pathogen and susceptibility of host (Frances *et al.*, 2006; Bonaterra *et al.*, 2003).

A biological control agent can inhibit the pathogen development by means of several mechanisms including antibiosis, direct interaction, competition for space and nutrients, quorum quenching and induction of plant resistance mechanisms. Nevertheless, it has been described that more than one mechanism might take part in the suppression of disease (Whipps, 2001), which may be affected by environmental conditions (Weller, 1988), making difficult to determine the relative importance of each mechanism and to find out the main mechanism responsible of biocontrol (Fravel, 1988). Thus, it is necessary to assess the role of each individual mechanism for determining the relative contribution to the whole biocontrol process (Wright and Beer, 1996).

1.1. Approaches to study biocontrol mechanism

Tools classically used to study mechanism of action are based on approaches, as they do not demonstrate that the mechanism studied was responsible of their ability to biocontrol.

The role of **antibiosis** as main biocontrol mechanism of the BCA has been object of many works, mainly it has been studied using *in vitro* assays (Winkelman *et al.*, 1980; Ishimaru *et al.*, 1988; Vanneste *et al.*, 1992; Wodzinski and Paulin, 1994; Kearns and Hale, 1996; Chernin *et al.*, 1996; Wright *et al.*, 2001; Fogliano *et al.*, 2002; Kamilova *et al.*, 2005). For example, two strains of *Pseudomonas syringae*, ESC-10 and ESC-11, which are commercially available to control postharvest disease (Bull *et al.*, 1997), produce syringomycin E *in vitro*, a cyclic lipodepsinonapeptide that inhibits the pathogens *Geotrichum citriaurantii*, *Penicillium digitatum*, and *Rhodotorula pilimanae* when the strains were coinoculated on the same agar surface. Moreover, they observed that the purified antifungal compound from strains ESC-10 and ESC-11 also controlled green mould caused by *P. digitatum* on lemon, concluding that syringomycin E

produced by both strains may be involved in biological control of green mould on citrus. Edwards and Seddon (2001) have assessed the activity of *Brevibacillus brevis* (*Bacillus brevis*) and the antibiotic it produces, gramicidin S, against the plant pathogen *Botrytis cinerea in vitro* and *ex vivo* (leaf sections of Chinese cabbage) assays. The authors found that *B. brevis* and gramicidin S were highly sporicidial in *in vitro* assays. But, due to strong binding of the antibiotic to the leaf sections of Chinese cabbage, it was much less active *in planta* assays. Thus, the different activity of gramicidin S showed *in vitro* and on leaf sections indicates a BCA that inhibits pathogen by means of antibiosis *in vitro* may not maintain its control in field conditions. This result justifies the need to study mechanisms of action of a BCA with different assays including *in vitro* and bioassays more approximate to field conditions.

BCAs may compete with pathogens for nutrients and space delaying or preventing decay of fruit after harvest. These mechanisms have been difficult to study because no method is available to determine the significance of each component of competition on disease suppression. To evaluate **competition for nutrients**, Janisiewicz *et al.* (2000) have developed a simple approach to test the effect of nutrient depletion by an antagonist on germination and growth of the pathogen with an *in vitro* system closely resembled *in vivo* conditions. It is based on a non-destructive method using tissue culture plates with cylinder inserts containing a semipermeable membrane at the bottom part. This approach has been used by many authors to study competition for nutrients on postharvest diseases (Janisiewicz *et al.*, 2000; Poppe *et al.*, 2003; Bonaterra *et al.*, 2003). For example, Bonaterra *et al.* (2003) observed a significant inhibition of conidial germination and hyphal growth of *Rhizopus stolonifer* and *Monilinia laxa* when the fungal spores and *P. agglomerans* EPS125 cells were cocultivated on peel leachate or nectarine juice. However, this effect was not detected when the antagonist and pathogen cells were physically separated by the semipermeable membrane which allows nutrient and metabolite interchange. Therefore, unlike production of antibiotic substances or nutrient competition, direct interaction between strain EPS125 and pathogen cells is essential for biocontrol activity of antagonist strain EPS125. In this way, similar findings were obtained for the biocontrol agent *P. agglomerans* CPA-2 against *Penicillium digitatum* in where not only competition for nutrients was one of the modes of action, but also physical contact between pathogen and antagonist was important for the effective control (Poppe *et al.*, 2003).

In addition, the involvement of nutrient competition in the interaction of a BCA and pathogen of bacterial nature can be studied by means of the comparison of parameters obtained from growth-substrate concentration curves of the pathogen and antagonist as well as, determining the niche overlapping index (NOI), which analyzes spectrum of nutrient use and niche overlap of both microorganisms according to the number of carbon sources of Biolog GN plates utilized by biocontrol agent and pathogen (Janisiewicz, 1996). For example, the biocontrol agent of fire blight, *P. fluorescens* EPS62e, exhibited good nutrient competition ability according to NOI, as it used most of the carbon sources used by *E. amylovora* EPS101, and it presented a maximum growth rate (μ_{max}) and higher nutrient affinity (lower Ks) than *E. amylovora* on immature pear fruits juice (Cabrefiga, 2004). These results indicate that competition for nutrients may be a mechanism involved in the suppression of fire blight.

A biocontrol agent should have the ability to establish and grow in the same ecological niche as the pathogen. Without this characteristic the biocontrol agent cannot interact with the pathogen and develop an efficient mechanism of inhibition (Vanneste, 1996). For example it has been demonstrated that colonization of the stigmatic surface of flowers with non pathogenic bacteria such as *P. fluorescens* or *P. agglomerans* can greatly inhibit colonization by *E. amylovora*, leading to a substantial reduction of disease (Lindow and Andersen, 1996; Johnson and Stockwell, 1998; Pusey, 2002). To assess the implication of **competition for space** in biological control, there are necessary colonization studies of the BCA and the pathogen on the host tissue by means of microscopic analysis. For instance, through Scanning Electron Microscopy (SEM) analysis, the ability of *P. fluorescens* EPS62e to colonize and grow in fruit wounds and flowers

affecting *E. amylovora* infection could be observed. Therefore, pre-emptive colonization together with nutrient depletion and cell-to-cell interaction with pathogen cells may be involved in biocontrol of fire blight by *P. fluorescens* EPS62e (Cabrefiga, 2004).

Moreover, microscopic analysis is a powerful tool that gives information about other types of interactions between BCA and pathogen. For example, Bryk *et al.* (1998) showed through optical microscope observations that **direct interaction** mechanism was involved in biocontrol of postharvest pathogens *in vitro*. They found that *P. agglomerans* B66 and B90 cells were able to biocontrol *Botrytis cinerea* and *Penicillium expansum* pathogens through parasitic events such as taxis to the conidia and germ tubes and lysis of germ tubes in diluted apple juice.

Induction of Systemic Resistance (ISR) in plants against pathogen is another biocontrol mechanism described. To study this mechanism, several works have focused in systems where the inducing bacteria and the challenging pathogen remainded spatially separated during the experiment and no direct interaction between the bacteria and pathogen occurs (Sticher *et al.*, 1997; van Loon *et al.*, 1998). In this way, Kamilova *et al.* (2005) showed that *P. fluorescens* WCS365 induced resistance towards *Fusarium oxysporum* in tomato plants without direct contact with the pathogenic fungus.

Finally, **quorum quenching** has arisen as a novel biocontrol mechanism against bacterial pathogens recently. Quorum quenching is based on the production of AHL-lactonases and AHL-acylases enzymes by the biocontrol strain. These degrade *N*-Acyl homoserine lactones (AHLs) molecules which are responsible for triggering virulence genes via quorum sensing (Dong *et al.*, 2001; Bauer and Robinson, 2002; Zhang, 2003). For example, a study perfomed by Dong *et al.* (2004) reports how *Bacillus thuringiensis* suppressed the quorum-sensing-dependent virulence of plant pathogen *Erwinia carotovora* through the production of AHL-lactonases in *in vitro* and *in planta* conditions. *B. thuringiensis* interfered in the accumulation of AHL signal when they were cocultured *in vitro*, and it decreased the incidence of *E. carotovora* infection and symptom development of potato soft rot *in planta*. Other AHL-lactonase producing *B. thuringiensis* strains analysed also provided significant protection against *E. carotovora* infection unlike *Bacillus fusiformis* and *Escherichia coli* strains, which do not process AHL-degradation enzyme showing little effect in biocontrol. These results points out the direct correlation of biocontrol activity with the ability of bacterial strains to produce AHL-lactonases.

In summary, all these studies increase knowledge about mechanisms in biocontrol process, but they do not demonstrate which is responsible of its biocontrol ability. To improve understanding of antagonist-pathogen interaction, this information should be coupled with genetic studies about the gene or genes that conferred the biocontrol ability. Molecular tools based on the study of gene differential expression (mRNA differential display), induction of gene expression (IVET) or mutation of essential genes for pathogenesis or biocontrol, have been useful tools broadly used in the last years.

1.2. Genetic tools to study biocontrol mechanisms

A method developed by Liang and Pardee (1992), **mRNA differential display**, allows the identification and isolation of differentially expressed genes in several experimental systems. This method has shown to be powerful due to its simplicity and allows the simultaneous comparison of up- and down-regulated genes with few amounts of raw material. In this technique, the mRNA population is sampled by the production of randomly amplified DNA fragments using reverse transcription followed by amplification by PCR (RT-PCR) (Liang and Pardee, 1992; Liang *et al.*, 1993). In the original description of mRNA differential display, the synthesis of cDNA molecules from mRNA by reverse transcriptase is initiated by poly-dT primers that anneal to poly(A) tails of the message. This protocol cannot be applied to the discovery of prokaryotic genes because it relies on stable poly(A) tails which are absent in prokaryotes. A much less variant of mRNA differential display, RNA fingerprinting by arbitrarily

primed PCR (RAP-PCR), uses an arbitrary primer to initiate the reverse transcription step and can be applied to prokaryotes (Welsh and Herbert, 1999). However, only a few prokaryotic applications of differential display have been reported (Mahan *et al.*, 1993; Wong and Mcclelland, 1994; Zhang and Normark, 1996; Yuk *et al.*, 1998; Fleming *et al.*, 1998; Walters *et al.*, 2001; Bidle and Bartlett, 2001).

Nevertheless, to work with mRNA differential display method implies several drawbacks including false positives generated by the PCR step; contamination of bands recovered from display gel by heterogeneous sequences; identification of differentially expressed genes remains as a tedious and complicated task; difficulty to work with prokaryotic systems; amplified patterns obtained in *in vitro* assays are submitted to the conditions chosen implying a risk of not visualizing interesting bands; and finally, expression bands obtained in *in vitro* and *in vivo* assays are discarded for further analysis although they may be related with pathogen or biocontrol activity of microorganism.

In vivo expression technology (IVET) was the first practical strategy described for selecting bacterial genes expressed preferentially during infection of an animal host (Mahan *et al.*, 1993). The original description performed about IVET technique was based on the use of purine auxotrophs (purA) of *Salmonella enterica* serovar *Typhimurium* which were rapidly eliminated from the mouse unless they are complemented. With the aim to identify the in vivo-induced (*ivi*) genes related with its pathogenic activity, random genomic fragments were cloned upstream of a promoterless *purA-lacZY* synthetic operon present on a suicide vector. Then, this library was transferred by conjugation into purA *Salmonella* cells and was integrated by homologous recombination forming merodiploids, which increase the likelihood to identify essential *ivi* genes for survival and growth in the host. The *Salmonella* library was injected into the peritoneal cavity of mice, and systemic spread and growth in the mouse provided positive selection for strains carrying an *ivi* gene, which may be related with virulence, was driving expression of *purA-lacZY* operon. Similar IVET selections, incorporating *purA* or other selectable complementing genes, have been used to identify *ivi* genes in a large number of gram-negative and gram-positive pathogens, as well as in biocontrol agents (Rainey, 1999; Fuller *et al.*, 1999; Handfield and Levesque, 1999; Gahan and Hill, 2000; Lee and Cooksey, 2000).

The most significant disadvantage of IVET is that it discriminates perhaps too strongly against genes that are expressed *in vitro*, which are discarded to perform *in vivo* analysis. However, there is no reason to expect that *in vitro*-expressed genes would not be important for either survival in the host or to cause damage or to provide protection to the host (Chiang *et al.*, 1999). Another limitation of the IVET method is that it is relatively limited to bacteria with tractable genetic systems because of the requirement for high frequencies of homologous recombination and extensive strain manipulation before strain selection (Covacci *et al.*, 1997).

A sequenced genome may allow the identification of all proteins of a given organism, but this does not provide sufficient information about the pathways and structures in which those proteins function and contribute to biocontrol activity. To take full advantage of the sequence information, it is necessary to integrate it with the cellular and developmental biology of the organism. This integration requires functional analysis of genes that can be easily accomplished by mutation (Table 1.1). Classically, methods used for generating mutations have been nonspecific **spontaneous** and **induced mutagenesis**. These have the advantage that are inexpensive and are not limited to our current understanding of DNA sequences. However mutants obtained by induced mutagenesis, with chemical or UV treatments, present a low stability and their analysis could be complicated by the presence of multiple sites of mutation events. For overcoming this problem, the use of mutagenesis with **transposons** has become the most important alternative for generating a single mutation. This method not only allows **random** mutagenesis to identify sequences that may not have been identified by computer-assisted gene prediction methods and to detect unknown aspects of genome function, but also **signature-tagged mutagenesis** (STM) generating mutations in selected sequences such as

functional or regulation system genes. Therefore, in case of biological control agents, random mutagenesis with transposons is a suitable tool to obtain information about sequences related with biocontrol mechanisms from unknown genomic sequences of BCA. Moreover, unlike mRNA differential display and IVET methods, mutagenesis with transposons allows the correct identification of biocontrol agents defective in biocontrol and the study of the respective impaired biocontrol genes from *in vitro* and *in vivo* assays.

Table 1.1. Examples of biocontrol agent mutants that have been generated to study their biocontrol mechanisms.

Mutation method	Microorganism	Gene	Characteristics of mutants	Reference
Spontaneous	*P. fluorescens* PCL1751	unknown	Mutant impaired in motility	Kamilova *et al.*, 2005
Induced (UV)	*Pseudomonas batumici* N17	unknown	Overproducer of batumin	Smirnov *et al.*, 2000
Transposon (Omegon)	*P. fluorescens* B16	unknown, ORF1-ORF4	Defective in antibiotic biosynthesis	Kim *et al.*, 2003
Transposon (Tn5)	*Erwinia herbicola* Eh252	unknown	Defective in antibiotic biosynthesis	Vanneste *et al.*, 1992
Transposon (Tn5)	*Pseudomonas* sp. WCS417r	*sid*	Defective in siderophore biosynthesis	Duijff *et al.*, 1993
Transposon (Tn5)	*P. chlororaphis* PCL1391	unknown (motility, auxotroph), *sss*	Impaired in the colonization traits: motility, amino acid prototrophy and site-specific recombination (Sss/XerC)	Chin *et al.*, 2000
Transposon (Tn5)	*Agrobacterium rhizogenes* K84	unknown, related *pvdD*	Hydroxamate siderophores	Penyalver *et al.*, 2001
Transposon (Tn5)	*P. agglomerans* Eh318	unknown	Defective in A and B pantocines biosynthesis	Wright *et al.*, 2001
Transposon (Tn5-*lac*)	*P. fluorescens* F113	unknown	Defective in 2,4-diacetylphloroglucinol biosynthesis	Cronin *et al.*, 1997
Transposon (Tn5-*lacZ*)	*Pseudomonas aureofaciens* 30-84	*phz*R	Phenazine regulator impaired lacking biosynthesis of PCN	Pierson *et al.*, 1994
Transposon (Tn5-*luxAB*)	*Pseudomonas chlororaphis* PCL1391	*phz*	Defective in phenazine-1-carboxamide (PCN) biosynthesis	Chin *et al.*, 1998
Transposon (Tn5- *luxAB*)	*P. chlororaphis* PCL1391	*phzI, phz*R	Defective in production of autoinducer molecules, C_4-HSL and C_8-HSL, lacking biosynthesis of PCN	Chin *et al.*, 2001

1.3. Mutagenesis with transposons

Characteristics and types of transposons

Transposable elements in bacteria were discovered in 1974 by Hedges and Jacob (1974). They found that whenever the gene for ampicillin resistance was transferred from one *E. coli* plasmid to another, the recipient plasmid always showed an increase in molecular weight of about 4.5 kb. Thus, they suggested that the gene for ampicillin resistance was carried on a discrete genetic element that could transpose, and they called this element **transposon**.

Transposons are between 2-20 Kb of size and present at least one gene which confers in the host bacterium a heritable property. The most common and intensively studied transposons carry genes conferring antibiotic resistance but other characteristics may also be encoded such as genes for arginine biosynthesis, heavy metal resistance or enterotoxin production. The main property of most of transposons is that have the ability to produce strong polar mutations in a random pattern remaining stable at their insertion sites. This mutation is produced by distortion of the open reading frame of the mutated gene, as well as several transcription terminators located on the transposon sequence (Lacy and Stromberg, 1995). As Table 1.2 shows, there is a large number of transposons described according to marker selection, reporter gene, specificity of insertion, size and polarity, but commonly based on Tn3, Tn5, Tn10 and Tn916 (Handfield and Levesque, 1999).

Table 1. 2. Examples of transposons carrying different genetic markers.

Transposon	Genetic markers	Reference
Tn1	Ampicillin resistance	Koekman *et al.*, 1980
Tn3	Ampicillin resistance	Pasquali *et al.*, 2005
Tn5	Kanamycin and neomycine resistance	Titarenko *et al.*, 1997; Penyalver *et al.*, 2001; Rojas-Jimenez *et al.*, 2005
Tn9	Chloramphenicol resistance	Bardonnet and Blanco, 1992
Tn10	Tetracycline resistance	Delorenzo and Timmis, 1994
Tn501	Mercury resistance	Falt *et al.*, 1996
Tn552	Kanamycin resistance	Colegio *et al.*, 2001
Tn916	Tetracycline resistance	DeAngelis, 1998
Tn1681	Enterotoxin (heat stable)	Fekete *et al.*, 2003
Tn3-HoHo1	lacZ reporter (β-galactosidase)	Stachel *et al.*, 1985
Tn5-1062	lux AB reporter (luciferase) Streptomycin and kanamycin resistances	O'Connell *et al.*, 2000
mini-Tn5	Nalidixic acid resistance	Kwon *et al.*, 1997
mini-Tn5 *lacZ2*	lacZ reporter (β-galactosidase) Kanamycin resistance	Delorenzo *et al.*, 1990
mini-Tn5 *luxAB*	lux AB reporter (luciferase) Tetracycline resistance	Delorenzo *et al.*, 1990
mini-Tn5 *xylE*	Catechol 2,3-dioxygenase reporter Kanamycin resistance	Delorenzo *et al.*, 1990
mTn5SS*gusA*40	GUS reporter (β-glucuronidase) Streptomycin and spectinomycin resistances	Wilson *et al.*, 1995; Aguilar *et al.*, 2002; Llama-Palacios *et al.*, 2003
EZ::TN<KAN-2>Tnp Transposome	Kanamycin resistance	Riess *et al.*, 2003; Qin *et al.*, 2004; Kawula *et al.*, 2004
Kan-mu transposomes	Kanamycin resistance	Lamberg *et al.*, 2002

Nevertheless, among all transposons, **Tn5** transposon has proven to be of great utility for the insertion mutagenesis of a variety of gram-negative bacteria (Delorenzo *et al.*, 1990). The generalised structure of Tn5 transposon is shown in Figure 1.1 and it consists of a central region usually encoding resistance to one or more antibiotics flanked by a pair of IS50 elements in inverted orientations (IS50L, IS50R). The IS50R is fully functional, it encodes the transposase protein which acts upon the terminal inverted repeat sequences to promote transposition. Some transposons (i. e. minitransposon GUS) do not have the *tnp* gene (IS50R) that encodes transposase in their construction but it is carried by the delivery suicide vector. In this way, the transposon maintains stable in its insertion point after transposition event. The Tn5 transposon duplicates a 9 bp of target sequence when it inserts at a new position. The frequency of transposition of each transposon varies from one element to another, and in case of Tn5, it shows a frequency of about 10^{-3} per element per bacterial generation.

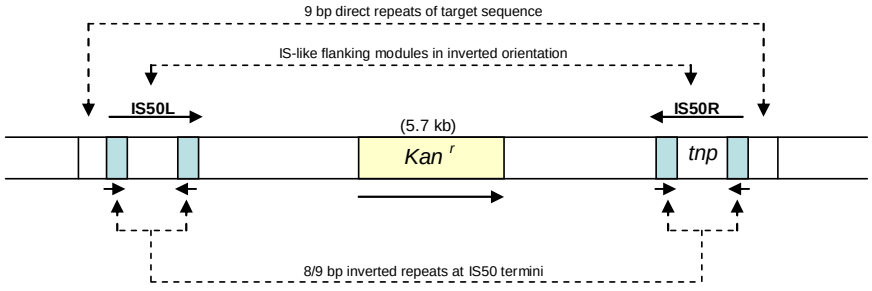

Figure 1.1. Structure of Tn5 transposon carying resistance to kanamycin.

Minitransposon is the generic name given to the members of a collection of genetic assets derived from transposons Tn10 and Tn5, in which the naturally occurring functional segments of DNA have been rearranged artificially to originate shorter mobile elements simplifying the generation of insertion mutants. This molecular nature facilitates the construction

of new mobile elements, in which not only new selective markers can be introduced (i. e. Sm, Km, Cm, Tc), but also a long variety of expression devices can be engineered for generating gene or reporter genes (i.e. β-galactosidase, β-glucoronidase, alkaline phosphatase, catechol 2,3-dioxygenase, GFP, *lux*/*luc*, *ice*/*ina*, surface reporters) (Table 1.2) (Delorenzo *et al.*, 1990).

Advantages and limitations of mutagenesis

The advantages and applications in genetic analysis of the powerful tool of mutagenesis with transposons are: (i) transposons can be used to create insertional knockout mutations; (ii) transposition can greatly facilitate genetic mapping providing genetic landmarks for the cloning of adjacent DNAs; (iii) they provide mobile restriction sites; (iv) transposons can be used to determine genetic physical maps; (v) transposons allow to generate gene-operon fusions to reporter functions; (vi) any bacterial gene can be mobilised and converted into a transposon and (vii) they locate primer binding sites for DNA sequence analysis (Goryshin *et al.*, 2000).

Nevertheless, some **limitations** of transposon mutational analysis have been described and are the following: (i) it might done non-random insertions; (ii) low frequency of transposition; (iii) lack of vector systems for specific genera, species, or strains of plant-associated bacteria; (iv) transformation, electroporation, transfection, or conjugation may not work or only work poorly with a specific bacterium; (v) it precludes the isolation of conditional lethal mutants; (vi) mutant phenotypes are not necessarily reflective of loss individual gene function, (vii) transposon insertion may trigger pleiotropic effects; and (viii) it is not possible to do complementation studies on individual genes in polycistronic operons (Ditta, 1986). However, in the last years novel transposons such as minitransposons and transposomes have been developed overcoming many of these difficulties such as the need of species-compatible transposase expression systems, transposition frequency and efficient transformation systems (Delorenzo *et al.*, 1990; Herrero *et al.*, 1990).

Selection of mutants

Once mutants have been obtained, it is important to choose an adequate screening program to select mutants that have altered the phenotype under study. In the case of biological control agents, it is necessary to decide the pathogen and the screening system (*in vitro, ex vivo, in vivo*). This screening program has to reproduce to more nearly approximate natural conditions that occur in plant or postharvest environment, as in these conditions the biocontrol agent employs its biocontrol mechanism to suppress disease. However, if there are evidences that a specific biocontrol mechanism is responsible of disease suppression, it is possible to perform a screening program focused it. For example, whether production of antimicrobial metabolites is suspected, a screening program based on *in vitro* assay will allow easily to select those mutants that have lost the ability to inhibit the pathogen by antibiosis (Wright *et al.*, 2001). However, lack of antimicrobial metabolites production by the mutant in *in vitro* assays does not necessarily mean this mechanism was responsible of biocontrol activity, as this mutant may maintain its inhibition capacity *in vivo* indicating other mechanisms are involved. For instance, Chin *et al.* (2000) selected three *P. chlororaphis* PCL1391 mutants impaired in colonization traits that did not control disease caused by *Fusarium oxysporum*, despite the fact that they showed normal antifungal activity *in vitro* and produced similar amounts of PCN than wild-type strain. For this reason, to improve selection of mutants of postharvest biocontrol agents, the screening program should be performed directly on the host tissue and in contact with the pathogen.

To analyze the phenotype of each mutant in a screening program, previously it is necessary to optimize the concentrations of the BCA and the pathogen, slowing down possibility of select false defective mutants or discard true defective mutants due to variability of the screening method. This purpose can be achieved through a previous study of dose-response effect with the wild-type strain, which provides information about the appropriate BCA population levels to inhibit the pathogen. In this way, those mutants with biocontrol activity

altered due to transposon insertion will be correctly selected for further studies. Moreover, dose-response assays also allow to determine quantitative parameters that describe the efficiency of the biocontrol agent permitting the comparison of different BCAs and pathosystems (Montesinos *et al.*, 1996; Larkin and Fravel, 1999; Bonaterra *et al.*, 2003).

Finally, parental strain and selected mutants are submitted to several phenotypic studies (growth, survival, colonization, metabolites production, etc) and genetic analysis (number of transposons inserted, sequencing of flanked regions of transposon, complementation of mutation) with the aim to approach and determine which mechanism or mechanisms are responsible of the disease suppression (Penyalver *et al.*, 2001; Aguilar *et al.*, 2002; Llama-Palacios *et al.*, 2003).

In conclusion, transposon mutagenesis is a useful tool for locating unknown genes that may be involved in BCA-pathogen-plant interaction. Specifically, it provides important information about gene or genes that may be responsible or related with biocontrol mechanism used by *Pantoea agglomerans* EPS125 to inhibit *Penicillium expansum* on apple.

2. Objectives

The purpose of this chapter was to determine the mechanism of action of *Pantoea agglomerans* EPS125 in the biocontrol of *Penicillium expansum* on apple fruit by means of phenotypic and genotypic studies of mutants impaired in their biocontrol activity obtained by transposon mutagenesis compared to the wild type strain EPS125.

3. Materials and Methods

Mechanism of action employed by *Pantoea agglomerans* EPS125 against *Pencillium expansum* on apple fruit has been analyzed by means of several phenotypic studies and using mutagenesis with transposons. The genetic manipulations consisted in the use of mutagenesis with the minitransposon GUS followed by phenotypic and genotypic analysis of mutants that were deficient in biocontrol activity.

3.1. Phenotypic evidences about mechanism of action

The determination of the biocontrol mechanism used by a BCA is difficult because usually more than one mechanism is involved and sometimes they are interrelated. Up to now, antibiosis has been the most studied mechanism due to it is easy to perform, and the competition for nutrients or space only has been implied in biocontrol indirectly due to the lack of appropriate methods to study.

In this section several approaches related to putative mechanism used by *P. agglomerans* EPS125 have been studied including production of antifungal metabolites, competition for nutrients, taxis of antagonist for pathogen, production of extracellular material by infrared (IR) analysis, observation of *P. agglomerans* EPS125-*Penicillium expansum* interaction in different conditions by means of Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM).

All assays of this section, with the exception of IR analysis and microscope observations, were performed twice maintaining the same experimental conditions to validate the obtained results.

3.1.1. Production of antifungal metabolites

The production of antifungal metabolites can be done with dual culture assays. The work performed by Vázquez (1998) showed that *P. agglomerans* EPS125 did not produce any diffusible metabolite against *P. expansum*. Nevertheless, these results can not be extrapolated to other conditions because they are submitted to the variable behaviour of biocontrol strain. For example, when a BCA colonizes fruit wounds it is exposed to different and more complex nutrient sources than in *in vitro* assays. Therefore, its metabolism and consequently its behaviour will be modified according to the environmental conditions. For this reason, in this section it was proposed to determine the production of antifungal metabolites of strain EPS125 in a system more similar to *in vivo* conditions using apple juice as nutrient source.

Preparation of apple juice

Two pieces of 'Golden Delicious' apples were grinded using a squeezer to obtain the culture medium of apple juice. Juice was filtered through a Whatman number 1 paper and sterilized by filtration with a filter of 0.2 μm of pore. The sterile apple juice was diluted to 5 % with sterile water and it was preserved at -80 ºC for long term storage in a deep-freezer.

Preparation of concentrated supernatants from spent cultures

Strain *P. agglomerans* EPS125 and two control strains, *Erwinia herbicola* EPS484 isolated from a *Pyrus malus* bud without biocontrol efficacy of *P. expansum*, and *Pseudomonas fluorescens* SBW25 given by Mark J. Bailey (Molecular Microbial Ecology, Institute of Virology and Environmental Microbiology, Oxford, England) that does not produce any secondary metabolite, were used in this section.

The three strains were grown in LB plates for 24 h at 23 ºC. After the incubation period, each strain was inoculated in 20 ml of sterile 1 % apple juice. A negative control based on sterile apple juice without inoculation was added. Cultures were incubated for 24 h at 23 ºC. Tubes were centrifuged at 3000 rpm for 15 min with a CR/CR3.12 centrifuge (Jouan, Barcelona, Spain) and supernatants were recovered in new sterile tubes.

Supernatants were dehydrated by freeze-drying with a temperature of -15 ºC in primary desiccation and 15 ºC in secondary desiccation; 300 mTorr of pressure; and a temperature of -45 ºC in the vacuum trap (Virtis, Gardiner, New York, USA). Freeze-dried products were suspended in 4 ml of sterile water concentrating 5 times all components of the apple juice (5X culture supernatant) and sterilized by filtration using a filter of 0.2 µm of pore size. In this way, putative metabolites present on spent juice with inhibition activity of pathogen germination will be detected.

Preparation of the pathogen

P. expansum conidia were obtained from a pure culture grown in potato-dextrose agar (PDA) plates for 7-8 days at 25 ºC without photoperiod. Spores were collected by scraping the culture surface with a wet cotton swab and resuspending the material in sterile distilled water containing Tween-20 (0.5 %). The conidia suspension was adjusted to $5x10^5$ spores/ml microscopically using a Thoma counting chamber (Brand, Germany).

Assay and analysis of results

Cell culture plates (Biolog Inc, Hayward, USA) and inserts of Millicell® tissue culture plates (Millipore, MA, USA) were used to determine whether concentrate supernatants of bacterial cultures inhibited the germination of *P. expansum* conidia (Figure 3.1). Inserts consist of a hydrophilic PTFE filter of 0.45 µm of pore size sealed to a cylindrical polystyrene holder. This filter allows the free interchange of medium nutrients and metabolites between the two compartments.

Figure 3.1. Cellular culture plate containing inserts of Milicell® tissue culture plates.

Each well of the culture plate was filled with 400 µl of 5X culture supernatant (EPS125, EPS484, SBW25), to a final concentration of 2X. A cylinder insert containing 100 µl of spore suspension adjusted to $5x10^5$ spores/ml (final concentration of $5x10^4$ spores/ml) was inserted to each well. Finally, all treatments were supplemented with 500 µl of 2 % apple juice (1% final concentration) to prevent for the lack of essential nutrients of the metabolized medium. The whole setup was incubated at 25 ºC for 13 h. Then, the cylinder inserts were removed and the membrane was blotted by the bottom side with tissue paper until all the liquid from the inside of the cylinder was absorbed. Thereafter, the membrane was recovered cutting with a sharp scalpel, transferred to a glass slide and observed under the microscope (Olympus optical co., Hamburg, Germany) at 200X to determine conidia germination. The assay was considered positive when >90 % of *P. expansum* spores germinated and negative when <1 % of spores were able to germinate.

3.1.2. Production of chitinases

With a sterile toothpick, strain EPS125 and the positive control *P. fluorescens* BL915 (provided by Steve Hill, Novartis Crop Protection, Inc. North Carolina, USA) were spread by puncture in plates containing colloidal chitin medium as described by Frandberg and Schnurer (1998) (annex I). Cultures were incubated for 5-6 days at 30 ºC. Strain was considered producer of chitinases when a clear halo around the colony appeared.

3.1.3. Competition for nutrients

The competition for nutrients between strain EPS125 and *P. expansum* was studied using the *in vitro* method described by Janisiewicz *et al.* (2000). In addition, two *ex vivo* variations of this method based on the use of apple fruit tissue similar to *in vivo* conditions were developed in this section.

Preparation of sterile apple juice, biocontrol agent suspension and pathogen suspension was performed as described in section 3.1.1.

In vitro assay

To test the effect of nutrient depletion by the strain EPS125 on germination and growth of the pathogen conidia, cell culture plates and inserts of Millicell® tissue culture plates were used.

Three different treatments were carried out (Table 3.1, Figure 3.2), in which 600 µl of 1.7 % apple juice was added in the well of the culture plate. The first treatment, "nontreated control", consisted of 400µl of the spore suspension of *P. expansum* at $1.25x10^5$ spores/ml inside the cylinder insert. The second treatment, "direct contact", consisted of a mixture of 200 µl of a bacterial suspension of EPS125 at $5x10^8$ cfu/ml and 200 µl of the pathogen suspension at $2.5x10^5$ spores/ml inside the cylinder insert. The third treatment, "separated", consisted of 200 µl of a bacterial suspension of EPS125 at $5x10^8$ cfu/ml in the well and 200 µl of the pathogen suspension at $2.5x10^5$ spores/ml inside the cylinder insert. The final concentration in all treatments were 10^8 cfu/ml for EPS125, $5x10^4$ spores/ml for *P. expansum* and 1 % for apple juice.

Table 3.1. Treatments performed to study competition for nutrients between the strain EPS125 and *P. expansum*.

Treatment	Well	Insert
Nontreated control	-	Fungal spores
Direct contact	-	EPS125+ fungal spores
Separated	EPS125	Fungal spores

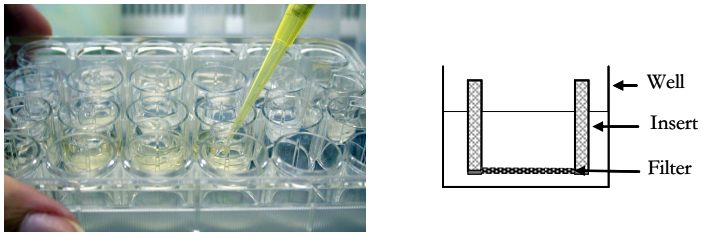

Figure 3.2. Setup of *in vitro* assay using apple juice as nutrient source for *P. agglomerans* and *P. expansum* for interaction studies.

Ex vivo assays

The pathogen and the biocontrol agent might not have the same accessibility to nutrients *in vitro* and *in vivo* conditions. For this reason, two types of *ex vivo* assays more approximate to *in vivo* conditions were developed. These were based on the use of cylinders of apple tissue and a whole apple fruit as nutrient source.

Cylinders of apple tissue were obtained with a flame sterilized cork borer of 1 cm of diameter and 1.5 cm of length. Cylinders obtained were deposited on the base of the well maintaining contact with the filter of the insert introduced later (Figure 3.3 A).

Whole 'Golden Delicious' apples were wounded with a cork borer producing a 'well' with similar dimensions of wells of cellular culture plates. Inserts were introduced into the well of apple maintaining direct contact with the fruit tissue (Figure 3.3 B). Inserts were covered with surrounded sterile cotton with gauzes to avoid contaminations during incubation period.

Three treatments (non-treated control, direct contact, separated) were performed following the Table 3.1. The same volumes and concentrations of pathogen and BCA used in the *in vitro* assay were maintained. The two *ex vivo* devices were incubated at 25 ºC for 13 h. Apple fruit also were maintained with high humidity conditions.

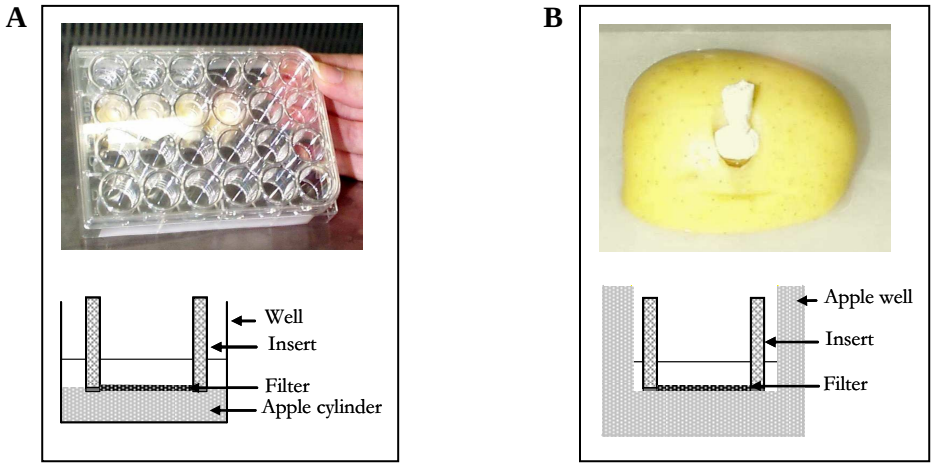

Figure 3.3. *Ex vivo* assay setups for interaction studies. (**A**) Apple disks as nutrient source, (**B**) An apple fruit half as well for placing insert and as nutrient source.

Analysis of results

The lecture of results of *in vitro* and *ex vivo* assays was obtained as described the previous section 3.1.1.

3.1.4. Taxis to spores of *P. expansum*

The taxis of *P. agglomerans* EPS125 to spores and germ tubes of *P. expansum* was studied in *in vitro* assays.

Preparation of pathogen and antagonist suspensions

P. expansum conidia were obtained from a pure culture in PDA plates for 7-8 days at 25 ºC without photoperiod. Spores were collected by scraping the culture surface with a wet cotton swab and resuspending the material in sterile distilled water containing Tween-20 (0.5 %). Suspension was centrifuged at 2000 rpm for 5 min at 4 ºC with the 5810R centrifuge (Eppendorf, Hamburg, Germany). Supernatant was discarded and pellet was washed twice with sterile distilled water and was resuspended in sterile phosphate buffer (annex I). The conidia

suspension was adjusted to 10^5 spores/ml microscopically by use of a Thoma counting chamber (Brand, Germany).

A single colony of *P. agglomerans* EPS125 was inoculated and incubated over night in LB broth. The culture was centrifuged at 4000 rpm for 10 min, supernatant was discarded and the pellet was resuspended in sterile phosphate buffer adjusting the concentration to 10^9 cfu/ml using a viable versus absorbance at 600 nm curve specific for *P. agglomerans* EPS125.

Assay and analysis of results

Two assays were performed to study taxis of biocontrol agent to germinated spores (first plate) and conidia (second plate) (Hawes, 1987).

A drop of spore suspension was deposited on one end of two plates containing 2 % agarose. The first plate was sealed and incubated at 25 ºC for 24 h allowing the germination of conidia. Once spores germinated, 20 µl of the antagonist suspension was deposited on the middle of the plate. In the second plate, the strain EPS125 was spread at the same time that pathogen. Plates were incubated at 25 ºC for 24 h.

The displacement of the antagonist to the pathogen was quantified measuring the distance between the biocontrol suspension and the pathogen upon incubation.

3.1.5. Analysis of production of exopolysaccharides by fourier transform-infrared spectroscopy (FT-IR)

FT-IR spectroscopy is a non-destructive technique that allows the rapid and qualitative characterization of structural features of polymeric material from IR spectra, which provide information regarding the particular types of vibration and rotational motions of atoms in molecules. Specifically, FT-IR spectrometry has been used to study and identify molecular structures that have been synthesized by *P. agglomerans* EPS125 in three conditions, in osmoadapted medium, LB broth and apple juice.

In the first treatment, a preculture of EPS125 in osmoadaptation medium of glucose semidefined medium (SDM) (annex I) was prepared to induce exopolysaccharide production. Then, 5 ml of SDM medium was inoculated with the preculture. In the second and third treatments, a single colony of EPS125 was inoculated in 5 ml of LB broth and 5 ml of sterile 1% apple juice. The three cultures were grown in an orbital shaker at 23 ºC for 24h at 150 rpm.

A drop of each culture was deposited on a clock glass and was dried in a vacuum desiccator at 25 ºC using silica gel as desiccant. For FT-IR analysis, the dry material was mixed with dry potassium bromide and was ground into a fine powder using an agate mortar before compressing into the KBr disk under a hydraulic press at 10,000 psi. Each disk was scanned over a wave number region of 400 to 4000 cm^{-1} with a Galaxy series FTIR 5000 (Galaxy series FTIR 5000, Mattson). The frequency-dependent absorption patterns that describe the structure of the polymeric material present on samples were recorded. IR absorbance bands present in IR spectra were used to identify molecules by means of comparison with patterns described on bibliography.

3.1.6. Interaction studies using scanning electron microscopy

In vitro and *ex vivo* assays were performed to study the interaction between biocontrol agent and pathogen by means of scanning electron microscopy (SEM). The *in vitro* assay took place in inserts of Millicell® tissue culture plates having apple juice as nutrient source. The *ex vivo* assay was based on the use of a wound produced on apple fruit as well and nutrient source. The same procedure and concentrations of BCA and pathogen showed in "3.1.2. Competition for nutrients (*in vitro*)" section were used, but following the treatments and incubation periods

indicated in the Table 3.2. In the *ex vivo* assay, a new treatment consisted of pathogen inoculation in apple wound 24 h before the treatment with EPS125 was added.

Table 3.2. Treatments and incubation periods used to study interaction between the strain EPS125 and *P. expansum* by means of SEM.

Treatment	Insert or wound contents (µl)	Incubation period (h)
In vitro		
Nontreated control	Fungal spores (400)	1, 4, 8, 13 and 15
Direct contact	EPS125 (200) + fungal spores (200)	13
Ex vivo		
Nontreated control	Fungal spores (400)	13
EPS125 control	EPS125 (400)	13
Direct contact	EPS125 (200) + fungal spores (200)	13
Direct contact germinated	EPS125 (200) + fungal germ tubes (200)	24 (germination period), 13

Sample processing

Samples (inserts and apple wounds) were processed according to conventional protocol for SEM. These were fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer (pH 7.1-7.4) for 2 h at 4 ºC and washed with 0.1 M sodium cacodylate buffer. Then, they were dehydrated in a graded ethanol series (50, 70, 90, 96 and 100%) and critical point dried with CO_2 in a pressure pump (K850, Emitech, England). In this point, filters were recovered and apple wounds were cut by its equatorial plane. Specimens were mounted on metal stubs with colloidal silver (Electrodag 1415, Acheson, Holland) followed by gold sputter coat in a sputter system (K550, Emitech, England). The observations were done with a SEM with 3.5 mm of resolution and at an accelerating voltage of 15 kV (DSM 960A, Zeiss, Germany). Image capture and measurements were accomplished using a digital image acquisition system by Quartz PCI program (Version 5.1, Quartz Imaging Co., Canada). Sample processing and SEM and TEM observations were performed in the Servei de Microscopia dels Serveis Integrats de Recerca de la Universitat de Girona (Girona, Spain).

3.1.7. Interaction studies using transmission electron microscopy

Transmission Electron Microscope (TEM) observations were performed to view extracellular structures and saccharic components of *P. agglomerans* EPS125, such as lipopolysaccharide, capsule and extracellular glycoproteins stained with ruthenium red.

The EPS125 was grown in 2 ml of LB broth and 10% apple juice at 23 ºC for 24 h. Cultures were fixed in a 2.6 % gluteraldehyde and 0.05 % ruthenium red in 0.1 M sodium cacodylate buffer (pH 7.1-7.4) for 4 h at 4 ºC. Suspensions were washed with the same buffer and pellets were suspended in 2% (w/v) Noble agar (Difco) and repeatedly washed in cacodylate buffer. Post fixation was done in 2 % osmium tetroxide and 0.05 % ruthenium red in 0.1 M cacodylate buffer and dehydration in a graded acetone series. Dehydrated samples were embedded in epoxy resin according to Spurr (1969) protocol. Resin blocks were cut in semithin sections (1-2 µm) with a glass knife ultramicrotome (OMU-2, Reichert, Austria). These sections were examined with a light microscope (DMXR-XA, Leica, Germany) to locate the bacterial accumulations. A negative control based on a suspension of EPS125 grown in apple juice but did not stain with ruthenium red was added.

For electron microscopy, ultrathin sections (60-90 nm) from resin blocks were cut with a Diatome diamond knife ultramicrotome (OMU-2, Reichert, Austria). Sections were collected on 200 mesh copper grids. The grids were placed in 2 % uranyl acetate for 30 min, followed by Reynold's lead citrate for 10 min. The stained sections were examined with a TEM operating at 60 kV (EM 910, Zeiss, Germanny). Image capture was performed by photo negatives on the

TEM using an electron microscope film (4489, Eastman Kodak Co., NY, USA) 8.3x 10.2 cm sheet.
In addition, an overnight culture of EPS125 grown at 23 ºC in 10 % apple juice was processed according to negative stain protocol. A volume of 2 ml was centrifuged at 3000 rpm for 10 min and the supernatant was discarded. Pellets were washed twice with 1 ml of 100 mM Tris, 150 mM NaCl buffer pH 7.6 and concentred in 200 µl of buffer. The suspension was deposited on 200 mesh copper grids and it was let rest during 3 min. Water excess was recovered with a filter paper. Samples were negative stained with 2 % uranyl acetate for 3 min and then, the remainder reactive was removed. Samples were examined with a TEM operating at 60 kV. Image capture was performed by photo negatives on the TEM using an electron microscope film (4489, Eastman Kodak Co., NY, USA) 8.3x 10.2 cm sheet.

3.2. Mutagenesis with transposons and mutant characterization

3.2.1. Design of mutagenesis

The purpose of this section was to determine mechanism of action of *P. agglomerans* EPS125 in the biocontrol of *P. expansum* on apple fruit by means of phenotypic and genetic studies of mutants defective in biocontrol activity obtained by random mutagenesis with the minitransposon GUS.

Bacterial strains, media and growth conditions

Table 3.3. Bacterial strains and plasmids used in this work.

Strain or plasmid	Relevant characteristics[a]	Reference or source
E. coli		
DH5α	*supE44* Δ*lac U169 (Ø80 lacZM15) hsd*R*17 recA1endA1gyrA96 thi-1relA1*	Hanahan, 1983
S17.1-λ*pir*	294::[RP4-2 (Tc::Mu)(Km::Tn7)] *pro res* Δ *recA,* Tp^r, mod+, λ*pir*	Delorenzo and Timmis, 1994
JB525	MT102: *araD139 (ara-leu)* 7697 Δ *lac thi hsd*R. pJBA132: pME6031 carrying *lux*R-P$_{luxI}$-RBSII-*gfp*(ASV)-T$_0$-T$_1$; Tc^r; most sensitive to *N*-(3-oxohexanoyl)-L-HSL	Andersen *et al.*, 2001
XL1-Blue	*endA1 recA1 gyrA96 thi hsdR17*(r_{K^-} m_{K^+}) *relA1 supE44 lac* [F' *proAB*$^+$ *lacI*^{q}ZΔM15 Tn*10*(Tet^r)]	Stratagene Corp.
P. agglomerans		
EPS125	Wild-type strain (Cb^r)	Vázquez, 1998
EPS125Rif^r	Wild-type strain (Cb^r; Rif^r)	Frances, 2000
m40	mutant of EPS125; Tn5 Spc/Sm^r	This work
m439	mutant of EPS125; Tn5 Spc/Sm^r	This work
m622	mutant of EPS125; Tn5 Spc/Sm^r	This work
m1210	mutant of EPS125; Tn5 Spc/Sm^r	This work
m2002	mutant of EPS125; Tn5 Spc/Sm^r	This work
m2126	mutant of EPS125; Tn5 Spc/Sm^r	This work
m4015	mutant of EPS125; Tn5 Spc/Sm^r	This work
Serratia liquefaciens		
MG1	Acyl-HSL producer	Givskov *et al.*, 1988
Chromobacterium violaceum		
CV026	Acyl-HSL-negative mutant of ATCC 31532; most sensitive to *N*-hexanoyl-L-HSL	McClean *et al.*, 1997
Plasmids		
pBluescript II SK(+)	Amp^r	Stratagene Corp.
pCAM140	mTn5SS*gus A40* Spc/Sm^r Amp^r	Wilson *et al.*, 1995
pBBR1-MCS3	pBBR1MCS derivative, Tet^r	Kovach *et al.*, 1995

[a] Tc or Tc^r, tetracycline resistance; Km, kanamycin resistance; Cb^r, carbenicillin resistance; Rif^r , rifampicin resistance; Spc/Sm^r, spectinomycin and streptomycin resistances; Amp^r, ampicillin resistance.

The bacterial strains and plasmids used in this study are listed in Table 3.3. Strains of *Escherichia coli* were cultivated at 37 ºC in LB medium. Strains of *P. agglomerans* were grown at 23 ºC in LB medium and in AB minimal medium (Chilton *et al.*, 1974) supplemented with 0.2% mannitol (ABM) as the carbon source (annex I).

Mutagenesis system

The minitransposon mTn5SS*gusA40* of 4 kb size includes the *Sm/Spc* gene conferring resistance to spectinomycin and streptomycin antibiotics, and the *gusA* gene encoding β-glucuronidase (GUS) which contains a strong Shine-Dalgarno translation initiation context, but not promoter, and thus it acts as a promoter-probe transposon (Figure 3.4) (Wilson *et al.*, 1995).

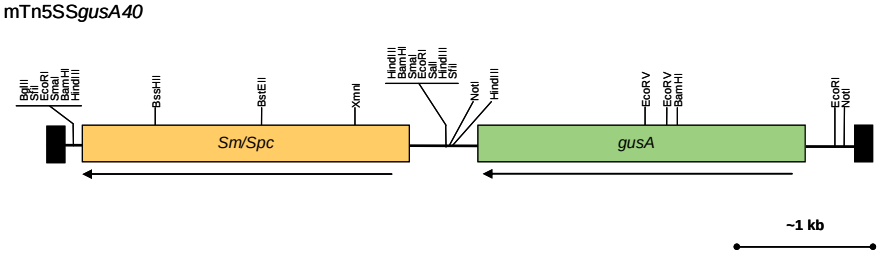

Figure 3.4. Restriction map of the promoter-probe minitransposon mTn5SS*gusA 40*. The antibiotic resistance cassette is flanked by transcriptional and translational terminators indicated as black rectangles.

This minitransposon is carried by the plasmid pCAM140, a R6K-based suicide delivery plasmid that provides the transposase (*tnp* gene) and whose conjugal transfer to recipients is mediated by RP4 mobilization functions in the donor (Handfield and Levesque, 1999). The transposase determined *tnp* gene is present nearby but outside the mobile element (Figure 3.5). This situation results in the loss of the *tnp* gene after insertion event, maintaining the minitransposon stable in the recipient genome and avoiding possible DNA rearrangements or other forms of genetic instability (Delorenzo *et al.*, 1990; Delorenzo and Timmis, 1994). A critical feature of the minitransposon is the system to deliver into the target strain. Specifically, pCAM140 is based on the R6K narrow-host range plasmid, which has the R6K origin of replication that requires the R6K-specified π protein. Therefore, this type of delivery plasmids are only maintained stably in λ*pir* lysogens or in *E. coli* strains with the *pir* gene recombined in their chromosome, such as *E. coli* S17.1-λpir (Delorenzo and Timmis, 1994). For mobilization by conjugation of pUT plasmids, where pCAM140 plasmid is included, these are carried by an *E. coli* strain with a chromosomally integrated RP4 that provides conjugal transfer functions which act on the *oriT* sequence present in the plasmid (Delorenzo and Timmis, 1994).

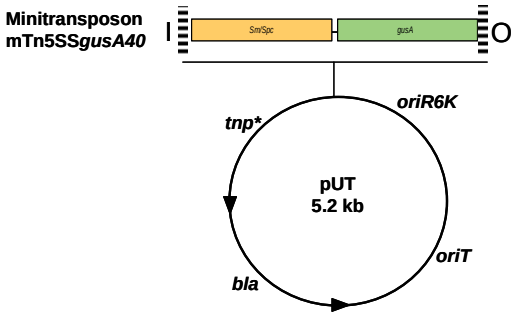

Figure 3.5. Scheme representing the construction of pCAM140 suicide delivery plasmid containing: *tnp* gene of Tn5 placed outside the mobile element; *bla* gene codifying for resistance to ampicillin; transference origin *oriT*; origin of replication *oriR6K*; and the minitransposon carrying Sm/Spc and GUS genes flanked by the two 19 bp I and O termini of Tn5.

Mutagenesis procedure

Minitransposon mTn5SS*gusA40*, which contains a promoterless reporter gene GUS and encodes for resistance to streptomycin (Sm) and spectinomycin (Spc), was introduced into strain EPS125 by conjugation via the suicide plasmid pCAM140 contained in *E. coli* S17.1-λpir. Strain *E. coli* S17.1-λ*pir* (pCAM140) was provided by P. Rodriguez-Palenzuela (Escuela Técnica Superior de Ingenieros Agrónomos, Madrid, Spain).

Donor strain *E.coli* S17.1-λ*pir* (pCAM140) was grown in LB agar culture medium supplemented with Spc (50μg/ml) and Amp (100 μg/ml) at 37 ºC for 24h. After this period, donor strain was grown in 6 ml of LB broth supplemented with spectinomycin (50μg/ml) and ampicillin (Amp) (100 μg/ml) at 37 ºC with shaking for 24h. *P. agglomerans* EPS125 was grown in LB agar culture medium at 23 ºC for 24h. A single colony of the recipient strain was grown overnight in 5 ml of MG/L medium (annex I) culture at 28 ºC with shaking. Two tubes containing 2 ml of donor strain culture and one tube with 2 ml of recipient strain were centrifuged at 13000 rpm for 10 min. Supernatants were discarded and pellets were washed twice with 1 ml of sterile AFT (annex I). A total of 2 ml of donor strain were collected in one tube. Tubes were centrifuged at 13000 rpm for 10 min. Supernatants were discarded and pellets were suspended in 1 ml of 10 mM SO_4Mg.

Three matings were performed as following, 500 μl of recipient strain *P. agglomerans* EPS125 and 200 μl of donor strain *E.coli* S17.1-λ*pir* were deposited on a sterile GTP filter of 0.22 μm of pore size and were filtered using a porcelain cap coupled to a XF5423050 vacuum pump (Millipore, MA, USA). In addition, two negative controls (three filters per control) were performed following the same procedure, but one with only the donor strain and another with the recipient strain. The resulting nine filters were deposited on plates containing NA medium (annex I) in up position and were incubated at 26 ºC for 16, 19 and 23h approximately. Three filters incubated for 12 h, corresponding to the mating and 2 controls, were suspended by shaking in 1 ml of sterile 10 mM SO_4Mg. Three consecutive decimal dilutions of each suspension were performed with AFT buffer and 100 μl of each dilution were spread on plates containing AB minimal medium supplemented with 0.2% mannitol (ABM) (annex I) and Spc (25 μg/ml) and Sm (50 μg/ml). A negative control based on *E.coli* S17.1-λ*pir* also was included (it cannot growth on minimum medium as it is proline auxotroph). Plates were incubated 72h at 37 ºC. The remaining suspensions were maintained at 4 ºC. The same procedure was followed for filters of matings and controls incubated 18 h and 24 h. If transconjugant colonies were obtained in all dilutions tested, it was proceed to the spread of the remaining suspensions of mating on plates containing ABM medium supplemented with Sm (50 μg/ml). Plates were incubated for 72 h at 26 ºC. Afterwards, two purification steps growing colonies on fresh plates containing ABM medium supplemented with Spc (25 μg/ml) and Sm (50 μg/ml) were performed. Finally, the collection of transformants obtained was stored for long term storage at -80 ºC in LB supplemented with 20 % of glycerol using microtiter plates of 96 wells (Figure 3.6).

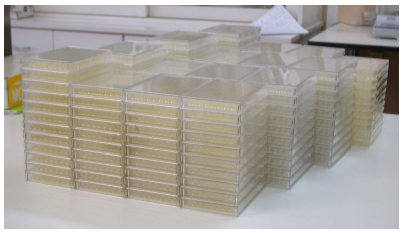

Figure 3.6. Collection of mutants stored in microtiter plates at – 80 ºC.

3.2.2. Selection of mutants

To select mutants impaired in their biocontrol activity it was necessary to develop a screening method with appropriate biocontrol agent and pathogen concentrations of application. Moreover, and if it is possible, it has to reproduce to more nearly approximate natural conditions where biocontrol agent employs its mechanism of action against the pathogen development. For these reasons, the screening program chosen in the present work was based on the selection of mutants impaired in their ability to inhibit disease caused by *P. expansum* on apple fruit following

the procedure described in '3.1.6. Dose-response assays (Chapter I)' section but inoculating 10µl of a *P. expansum* suspension at $5x10^4$ spores/ml and treating with 50 µl of bacterial suspension at 10^9 cfu/ml.

Preparation of bacterial suspension

4032 mutants were recovered from microtitre plate using a replica plater (Sigma-Aldrich Chemie, Steinheim, Germany) and were grown on plates containing ABM medium supplemented with Spc (25 µg/ml) and Sm (50 µg/ml) for 24 h at 23 ºC. Strain EPS125 and each mutant was overnight grown at 23 ºC in a tube containing 100 µl of LB medium and in case of mutants it was supplemented with Spc (25 µg/ml) and Sm (50 µg/ml). Tubes were centrifuged at 4000 rpm for 10 min and pellets were suspended with 1 ml of phosphate buffer to obtain a suspension about 10^9 cfu/ml.

Selection of mutants

'Golden Delicious' apple fruit were surface disinfected following the procedure described in "dose-response assay" section. Three processes of selection or screenings were performed following the scheme showed in Figure 3.7 (S1, S2 and S3). In the first screening, about 700 apples were wounded (nine wounds per fruit) with a nail tip to an approximate depth of 5 mm and a wide of 3 mm. Then, 7 wounds of each apple were treated with 50 µl of a 10^9 cfu/ml bacterial suspension corresponding to: one wound for *P. agglomerans* EPS125 (negative control) and 6 wounds for 6 different mutants (one mutant per wound). Two remaining wounds were treated with 50 µl of sterile water (positive controls). Fruit were let stand for 1 h, and all 9 wounds were inoculated with 10 µl of pathogen suspension at $5x10^4$ spores/ml. Fruit were placed in polystyrene tray packs in boxes that were sealed with plastic bags to maintain high humidity and incubated at 20 ºC in a controlled environment chamber for 5 days. Mutants corresponding to wounds that exhibited a diameter of lesion similar to positive controls were selected to perform a second screening (Figure 3.8). The same methodology used in the first screening was followed to perform a second and a third screening. The experimental design of the second screening consisted of three replicates of 1 fruit per replicate with nine wounds per fruit. One wound for negative control, two wounds for positive controls and three wounds per mutant. Mutants that maintained the altered phenotype were selected. From this group, 12 mutants were picked and were submitted to the third screening. The experimental design consisted of nine replicates of 1 fruit per replicate with nine wounds per fruit. Each fruit contained a negative control, two positive controls and six different mutants (one mutant per wound). In this way, mutants were selected correctly for their disease control capacity. Finally, 7 mutants (m40,

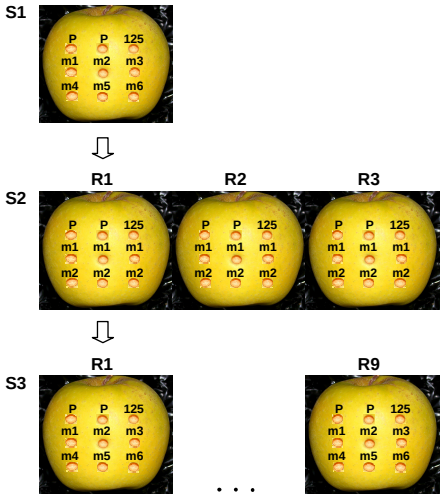

Figure 3.7. Scheme of the procedure followed to select mutants defective in biocontrol. **S:** Screening. **R:** Replicate. **P:** Positive control of pathogen. **125:** Negative control of pathogen inhibited by strain EPS125. **m:** Wound containing mutant and pathogen suspensions.

Figure 3.8. Selection of mutants in apple bioassays according to the lesion diameter caused by *P. expansum*. The two lesions in the left upper part of each apple fruit correspond to positive controls of *P. expansum* infection. It can be seen one mutant fully inactive in biocontrol activity (arrow).

m439, m622, m1210, m2002, m2126 and m4015) were selected to perform phenotypic and genetic studies about their impaired ability to suppress disease.

3.3. Characterization of mutants

3.3.1. Phenotypic studies of mutants

The 7 mutants selected (Table 3.3) were characterized by means of phenotypic assays providing information about: morphology of colonies, growth potential using LB as nutrient source, growth on apple fruit, expression of impaired genes in several conditions through promoterless GUS reporter gene, implication of signal molecules in quorum sensing systems, production of polysaccharides in several conditions and interaction with pathogen through SEM and TEM observations. Also, genetic analysis of disrupted sequence was performed with the aim to identify genes or gene products related with biocontrol mechanisms.

Mutants analyzed in this work were recovered from storage in ABM medium supplemented with Spc (25 μg/ml) and Sm (50 μg/ml).

Colony morphology

Morphology, mucus and colour of mutant colonies grown for 24 h at 23 ºC on plates containing ABM medium supplemented with Spc (25 μg/ml) and Sm (50 μg/ml) were performed by macroscopic observation.

Growth potential in culture broth

Growth curves of mutants referenced in Table 2.3. were compared with the parental strain *P. agglomerans* EPS125 through the Bioscreen system employing the concentration adjusted to 10^7 cfu/ml using a viable versus absorvance at 600 nm curve. A volume of 15 μl of each suspension was transferred into wells of a microtiter plate containing 185 μl/well of LB broth. Negative controls consisting of 200 μl fresh LB broth were included. Each treatment was repeated three times. Filled plates were placed in Bioscreen for analysis (Bioscreen C, Thermo Labsystem, Finland) and incubated for 48h at 25 ºC. Measurements were taken at 600 nm every 10 min with a prior shaking step to OD reading of 10 s.

The area under the growth curve (agc) and generational time (gt) values were calculated from absorbance data. A statistical analysis comparing each mutant with parental strain was performed and it consisted of mean separation of agc and gt variables through Tukey-Kramer test (P=0.05) with the general linear model (GLM) procedure of the PC-Statistical Analysis System version 8.2 (SAS Institute Inc., NC, USA).

Growth on apple fruit

The ability of EPS125Rif and the 7 mutants to survive and multiply in apple wounds was studied in this section. A volume of 50 μl of a suspension of each isogenic strain adjusted to 10^8 cfu/ml was applied to apples (cultivar Golden Delicious) which were previously disinfected and wounded with a cork borer as described above (3.2.2). Fruit were incubated at 13 and 23 ºC for 96 and 72 h respectively. Three replicates of 1 fruit per replicate were periodically sampled at 0, 24, 48, 72 and 96 h of incubation at 13 ºC and 0, 6, 12, 18, 24, 30, 48 and 72 h of incubation at 23 ºC. Samples were selected removing the fruit tissue containing the wound with a cork borer (10 mm diameter x 2 cm depth), placing the material in a sterile plastic bag with 20 ml of 0.1% peptone (annex I) and grounding with a pestle. The clear supernatant was serially diluted and the dilutions were seeded on LB agar plates supplemented with Rifampicin (Rif) (100 μg/ml) for strain EPS125 Rif; and Spc (25 μg/ml) and Sm (50 μg/ml) for mutants. Plates were incubated at 25 ºC for 24 h. Colonies were counted and data were transformed to $\log_{10}$ cfu/wound.

Population levels obtained from the 8 strains were analyzed to determine whether significant differences occurred. The effect of "strain" was determined by analysis of covariance (ANCOVA) followed by a multiple comparison through Tukey test in where the incubation time was considered as a covariate, using the GLM procedure of SAS.

Induction of GUS gene

Minitransposon mTn5SS*gusA40* contains a promoterless reporter gene GUS which allows to study the presence and induction of a promoter when it is placed in the correct orientation respect to the transposon insertion. Strain EPS125 and 7 mutants were seeded using sterile toothpicks in wells of microtiter plates containing 200 µl/well of LB broth and 1% apple juice supplemented with the substrate of the glucuronidase, X-GlcA stock (150 µg/ml) (annex I), and supplemented with Sm (50 µg/ml) in case of mutants. Each plate included a negative control inoculated with EPS125 and a positive control based on mutant m514, which showed a constant high signal of GUS gene expression in both assays. Each treatment was repeated three times. Then, filled plates were incubated for 48h at 37 ºC. GUS gene expression level was rated using a visual of increasing colour intensity as follows: -, suspension colour similar to negative control; (+), suspension colour bluish (juice) or greenish (LB); and +, blue (juice) or green (LB) suspension colour (Figure 3.9).

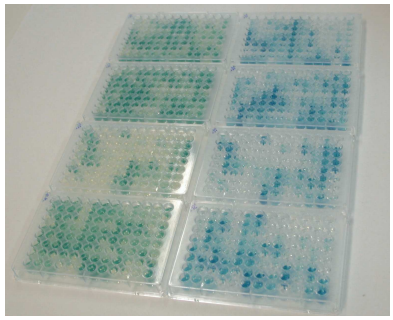

Figure 3.9. Induction of GUS gene of several mutants using LB broth and apple juice as nutrient sources.

Quorum sensing

Gram negative bacteria use a communication system via small diffusible N-acyl homoserine lactone (AHL) signal molecules. In this work, two bacterial sensor systems based on the macroscopic detection of violet pigment produced by *C. violaceum* CV026 and GFP by *E. coli* JB525 (Table 3.3), which were provided by C. Ramos Rodríguez (Facultad de Ciencias, Universidad de Málaga, Málaga, Spain), were used to detect the presence of AHL produced by parental strain EPS125 and the 7 selected mutants. Each strain was streaked close to AHL sensor bacteria (*C. violaceum* and *E. coli* JB525) to form a T approximately 5 cm and 5 cm high (Figure 3.10) in LB agar plates. A positive control using an AHL producer strain, *Serratia liquefaciens,* was included (Table 3.3). Plates were incubated at 28 ºC for 2-3 days. It was considered positive result when a violet halo around the *C. violaceum* colonies appeared and when a gfp fluorescence around *E. coli* colonies under UV light was observed, which was the result from the activation of its reporter genes by AHL molecules produced by tested strain.

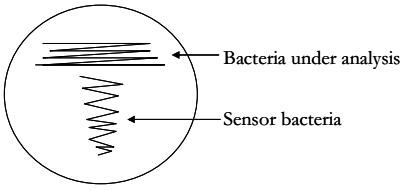

Figure 3.10. Scheme showing the relative position of bacteria under study and sensor bacteria.

Analysis of exopolymers by fourier transform-infrared spectroscopy (FT-IR)

FT-IR spectrometry has been used to compare absorption patterns of EPS125 with selected mutants when they were grown in LB broth and apple juice. The procedure was the following, 200 µl of a bacterial suspension of EPS125, m2126 and m4015 adjusted to 10^8 cfu/ml was transferred into a cylinder insert of Millicell® tissue culture plates, and 800 µl of LB broth into a well of cellular culture plate. Another assay was performed for EPS125 and 7 mutants maintaining the same procedure but using 1% apple juice as nutrient source. Plates were incubated at 23 ºC for 7 days changing daily nutrient source in both assays. Bacterial cultures were recovered and processed for IR as described in section 3.1.5.

SEM

In vitro and *ex vivo* assays were performed to study the interaction between EPS125 and 7 mutants (Table 3.3) with *P. expansum* by means of SEM.

The *in vitro* assay was performed using inserts of Millicell® tissue culture plates and cell culture plates. Wells of cellular culture plates were filled with 600 µl of 1.7% apple juice (1% final concentration) and inserts with a mixture of 200 µl of a bacterial suspension at $5x10^8$ cfu/ml (10^8 cfu/ml final concentration) and 200 µl of the pathogen suspension at $2.5x10^5$ spores/ml (final concentration of $5x10^4$ spores/ml). Plates were incubated at 23 ºC for 13 h.

To perform the *ex vivo* assay, Golden Delicious apples were disinfected, wounded and inoculated with the same volumes and concentrations of pathogen and bacterial suspensions described in the *in vitro* assay following the procedure used and described in section 3.2.2. Apple fruits were incubated at 23 ºC for 5 days.

The *in vitro* and *ex vivo* samples were processed and observed with SEM as described in section 3.1.6.

TEM

Observation of samples cut in ultrathin sections and negative stained were performed by means of TEM.

On one hand, *P. agglomerans* EPS125 and m2126 were grown in 10 % apple juice at 23 ºC for 23 h and were processed and ultrathin sections were examined as described in section 3.1.7.

On the other hand, overnight cultures of EPS125, m2126 and m4015 grown at 23 ºC in 10 % apple juice were negative stained following the protocol described in section 3.1.7.

3.3.2. Genetic analysis of mutants

Detection of the pCAM140 by PCR method

After mating process, transconjugants were grown on ABM medium supplemented with Sm antibiotic to select those colonies that have incorporated the minitransposon. Some of these transformant colonies were seeded in ABM plates supplemented with Amp to verify that transposon was inserted into genome of the recipient strain and it did not maintain itself into the cell as plasmid. True mutants cannot grow because they did not have Amp resistance codified by *bla* gene of the suicide plasmid pCAM140. However, in this work true mutants were not possible to be tested by selective growth using ampicillin because the parental strain EPS125 is resistant to this antibiotic.

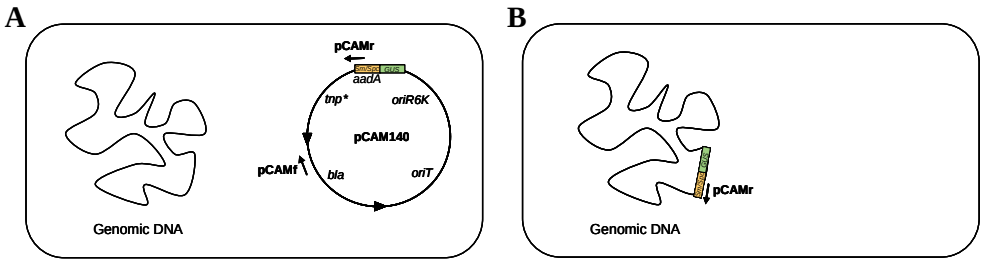

Figure 3.11. Scheme showing the relative position of primers (pCAMf and pCAMr) in two hypothetical situations. (A) Minitransposon has not jumped from pCAM140 plasmid to bacterial genome and therefore it will produce a fragment product of 2 kb by PCR method. (B) Minitransposon has inserted into bacterial genome and no amplification signal will be obtained because delivery plasmid has been degraded.

Nevertheless, a procedure to detect pCAM140 plasmid into cells by means of PCR was developed. This was based on the use of two specific primers designed from pCAM140 plasmid, a forward primer designed from *bla* gene (pCAMf) in the plasmid and a reverse primer from *aadA* gene carried in minitransposon (pCAMr) (Figure 3.11). Whether recipient cells have the plasmid structure, they will show a single amplified fragment of 2 kb of size, but if they have transposon inserted into the genome, it will not give amplification signal.

DNA extractions

Total DNA from *P. agglomerans* EPS125, *E. coli* S17.1-λpir (pCAM140) and 7 mutants (Table 3.3) was obtained following protocol described by Sambrook and Russell (2001). This starts from an overnight culture of 30 ml of each strain at an optical density of 1 OD at 620 nm. Cultures were centrifuged at 9000 rpm for 10 min and supernatants were discarded. Pellets were washed twice in 20 ml of 10 mM Tris, 10 mM EDTA and 150 mM NaCl (TEN) (annex I) and finally resuspended in 10 ml of TEN. Cells were incubated for 20 min at 37 ºC in the presence of 25 µl of a freshly prepared 50 mg/ml lysozyme solution and gently mixed. To each tube were added and gently mixed 33 µl of a freshly prepared 50 mg/ml proteinase k. Tubes were incubated at 37 ºC for 1h. Below, 1.1 ml of 10 % SDS were added and the mixture was incubated for 30 min at 50 ºC in a bath resulting a clear mixture. The same volume of saturated phenol (12 ml) was added and thoroughly mixed by tube inversion. Tubes were centrifuged at 9000 rpm for 15 min. Carefully, upper viscous phase containing DNA was transferred to a new tube and 12 ml of phenol-chloroform-isoamylic alcohol (25:24:1) were added. It was mix by tube inversion and centrifuged at 9000 rpm for 15 min. The upper phase was transferred to a new sterile tube. An equal volume of chloroform-isoamylic alcohol (24:1) was added. The upper aqueous phase was covered into a new tube and was carefully mixed with 0.1 volumes of NaAC 3M pH5.3. DNA was precipitated by addition of 2 volumes of ice-cold ethanol shaking carefully by hand. It may be incubated 30 min-overnight at -20 ºC or 30 min at -70 ºC. DNA strands were caught with a glass rod. DNA was washed in 500 µl of 70 % ethanol and resuspended in 500 µl of sterile water gently mixed. DNA solution was hydrated overnight at room temperature. The quality of DNA and the presence of RNA or nucleases in the preparations were checked by electrophoresis in a 0.8% agarose gel in 1X TAE buffer (annex I) at 65 V for 45 min. After this time, gel was stained with ethidium bromide (1 µg/µl) for 30 min and the PCR products were viewed with a UV transilluminator FX-20M (Vilvert Lournat, France). Pictures were digitally captured with a digital camera DC120 (Kodak, Madrid, Spain) connected to a computer and their processing were performed with the software 1D Image analysis system 120 (Kodak, Madrid, Spain).

RNA molecules were removed from DNA preparations by digestion with 7.5 µl of 10 mg/ml RNAse for 40 min at 37 ºC. DNA was recovered with phenol-chloroform-isoamylic alcohol (25:24:1) and chloroform-isoamylic alcohol (24:1) and it was precipitated and rehydrated in 100 µl of sterile water following the previous procedure. The quality and concentration of DNA preparations were determined using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and by electrophoresis.

In addition, pCAM140 plasmid was extracted from the strain *E. coli* S17.1-λ*pir* using the Plasmid extraction Wizard Plus SV Minipreps DNA purification system (Promega Corp., Tokyo, Japan) following the manufacturer's indications.

PCR conditions

A PCR reaction from genomic DNA of *P. agglomerans* EPS125, *E. coli* S17.1-λpir (pCAM140) and 7 mutants, and plasmid DNA (pCAM140) of *E. coli* S17.1-λpir was performed.

Two primers, pCAMf and pCAMr, were designed using Primer Express™ software (PE Applied Biosystems, MA, USA) to amplify the region comprised between *bla* (Amp resistance) and *aadA* (Sm/Spc resistance) genes of pCAM140. The pCAMf primer (5' TCTGGGTGAGCAAAAACAGG 3') was targeted to the *bla* gene presenting the pUT cloning

vector (pUT-miniTn5-gfp-tet) and pCAMr (5' TCCACTACGTGAAAGGCGAG 3') to the *aadA* gene of pMK2016 cloning vector. Sequences of both genes are available in GenBank database with the accession codes AY364166 and AY423864 respectively.

Amplification reaction was performed in a volume of 50 µl containing 0.4 µM of each oligonucleotide (Roche diagnostics, Manheim, Germany), 1.3 mM of dNTPs, 2.5 mM of $MgCl_2$, 1X buffer, 2.5 U of Taq polymerase (Invitrogen Life technologies, California, USA) and 10-20 ng of DNA extraction product. Amplification reactions were performed in a Gene Amp ® PCR System 9700 termocycler (PE Applied Biosystems, MA, USA). The PCR program consisted of an initial denaturation at 94 ºC for 4 min followed by 30 cycles consisting of 94 ºC for 45 s, 60 ºC for 45 s, 72 ºC for 2 min, followe by a extension step at 72 ºC for 7 min.

PCR products and λ*HindIII* marker at 50 ng/µl (Invitrogen Life technologies, California, USA) were separated by horizontal electrophoresis on a 1.5 % agarose gel in 1X TAE buffer at 65 V for 45 min. Gel was stained, visualized and images captured as it described above.

Determination of the number of transposon insertions

The number of transposon insertions in each mutant was determined by southern blotting and hybridization with two probes corresponding to Sm/Spc and GUS genes following standard protocols described by Sambrook and Russell (2001).

Southern blotting

Phenolized DNA (1 µg) of EPS125 and 15 mutants (40, 439, 622, 789, 1210, 2002, 2126, 2196, 3152, 3300, 3427, 3469, 3856, 3891, 4015) was digested overnight at 37 ºC with 15 U of *AccI* and 30 U of *SpeI* (Roche diagnostics, Manheim, Germany), restriction endonucleases that do not cut minitransposon. Digestion products and λ*HindIII* marker were charged and their DNA fragments separated by electrophoresis on 0.7% agarose gel. Gel was stained with ethidium bromide and photographed with a ruler laid along-side allowing the identification of band positions on the membrane. Below, southern blotting was performed following the scheme shown in Figure 3.12. Gel was immersed in 200 ml of denaturation solution for 45 min with shaking. It was rinsed with distilled water and was immersed in 200 ml of neutralization solution for 30 min with shaking. It was rinsed again with distilled water and was immersed in 200 ml of neutralization solution for 15 min with shaking. Gel was rinsed with distilled water and was immersed in 10XSSC (annex I) for 20 min with shaking. A solid support was placed on top of a plastic dish filled with enough 20XSSC (annex I) and covered with a Whatman 3MM paper wick, slightly larger than the gel in such a way that two ends were submerged in buffer. The agarose gel was placed on the filter paper and the edges were covered with four strips of plastic wrap. Nylon membrane (Amersham Biosciences, UK) was wet by floating on 2XSSC (annex I), submerging slowly, and then stand for 5 min. Membrane was placed on gel being careful to lay it down precisely the first time and avoiding bubble formation. Bubbles were removed by rolling a glass pipet over its surface. Membrane surface was flooded with 20XSSC. Three pieces of Whatman 3MM paper were cut to the same size as the membrane, wet with 2XSSC and placed on the membrane avoiding bubbles formation by rolling a glass pipet over its surface. Then, ~4 cm stack of paper towels with the same size as the membrane were layered on Whatman 3MM paper. The setup was covered with a glass plate and 500g weight and it was left transferring overnight. Paper towels and filter papers were removed. The membrane was marked with pencil to indicate the position of the wells. The up-down and back-front orientations were indicated by cutting one corner of the membrane. The nylon membrane was wrapped in UV-transparent plastic wrap, placed DNA-side-down on a UV light box and irradiated for 1200 x. The membrane was stored between sheets of Whatman 3MM paper for several months at room temperature.

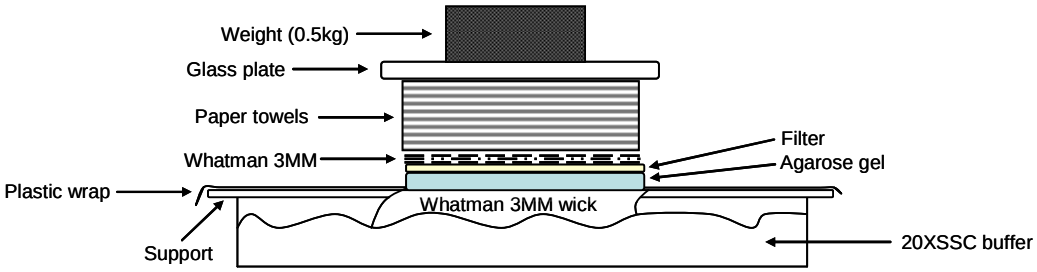

Figure 3.12. Southern setup used for upward capillary transfer of DNA from the agarose gel to the filter.

Marking of probes

Two probes consisting of Sm/Spc and GUS genes of minitransposon were radioactively labeled by random primed DNA labelling in the suitable installations pertaining to the Departamento de Biotecnología placed in Escuela Técnica Superior de Ingenieros Agrónomos (Madrid, Spain). Both genes were obtained by digestion of pCAM140 plasmid with *EcoRI* enzyme (Figure 3.3). Digestion reaction was performed in a volume of 20 µl containing 1X H buffer supplied with the enzyme, 20 U of *EcoRI* enzyme (Roche diagnostics, Manheim, Germany) and 1 µg of DNA (purified pCAM140 plasmid). The reaction was incubated at 37 ºC for 1 h and digestion products were separated in a 1.5% electrophoresis gel. Fragments corresponding to GUS gene (~2.3 kb) and Sm/Spc gene (~2 kb) were extracted from agarose gel and purified with Qiaex II Handbook kit (Qiagen GmbH, Hilden, Germany) following manufacturer' indications.

Then, purified DNA was denatured into single strands by incubation at 95 ºC for 5 min and annealed to random hexamer oligonucleotides. Random primers were extended using Klenow enzyme incorporating dTTP, dGTP, dCTP nucleotides and $\alpha dATP_{32}$ nucleotide. This labeled nucleotide consisted of a dATP labeled with a radioisotope alpha phosphate P-32. Marking reaction was performed in a volume of 20 µl containing 1X of hexanucleotide mix (Roche diagnostics, Manheim, Germany), 50 µCi (3000Ci/mmol) of α $dATP_{32}$ (supplied by the research group of P. Rodriguez-Palenzuela), 0.2 mM of dNTPs, 1X Klenow buffer, 1.5 U of Klenow 5' -exo (Invitrogen Life technologies, California, USA) and 25-50 ng of denaturalized DNA. Marking reactions were incubated for 2 h at 37 ºC. Then, probes were purified using Quick Spin Column (Roche diagnostics, Manheim, Germany) following manufacturer's instructions.

Hybridization

The hybridization procedure with probes was carried out behind a plexiglass shield. The nylon membrane was placed in a thermal sealing plastic tube. A volume of 10 ml of prehybridation solution (annex I) was added to each tube and the top was put on. It was incubated at 65 ºC with shaking. It was added 250 µl of salmon sperm DNA (10 mg/ml) previously incubated for 5 min at 100 ºC. Tube was incubated at 65 ºC for at least 1h. A volume of 50 µl of probe was added to the tube containing nylon membrane. It was capped tightly and mixed by rotation overnight at 65 ºC allowing hybridization process. Several posthybridization washes were performed to remove non-bounded probe. Membrane was placed in 50 ml of wash solution I (annex I) and incubated at 65 ºC for 20 min with gentle shaking. The nylon membrane was washed with 50 ml of wash solution II (annex I) at 65 ºC for 15 min followed by a wash with 50 ml of wash solution III (annex I) at 65 ºC for 10 min.

Autoradiography

Nylon membrane was placed between two pieces of Whatman 3MM paper to remove excess of wash solution. It did not allow the membrane to become completely dried. The semi-dry

membrane was placed in blot bags avoiding bubbles and placed asymmetrically for orientation purposes. Then, the nylon membrane was deposited and closed in a film holder (carboard Kodak X-ray film exposure holder). And in a darkroom, an X-ray film was placed between the intensifying screen and the blot bag. The cassette holder was closed and stored in the dark at -80 ºC for 5h. The exposure time varies according to the probes used and the counts obtained. Cassette was removed from the freezer and thaw at room temperature for 15 min. Film was developed, and wells and fragments of the molecular marker were drawn with a pencil.

Sequencing transposon flanking regions

DNA flanked transposon was sequenced by means of total digestion of genomic DNA and subclonation of hybridized fragments in southern blot analysis in a pBluescript plasmid (pBS) (Figure 3.13). Standard molecular cloning techniques employed in this study were described by Sambrook and Russell (2001).

DNA manipulations

Genomic DNA was extracted from EPS125 and 15 mutants (m40, m439, m622, m789, m1210, m2002, m2126, m3152, m3300, m3427, m3469, m3856, m3891 and m4015) using phenol-chloroform-isoamylic procedure as described above. Then, 5 µg of DNA was digested at 37 ºC overnight with 25 U of *AccI* and 50 U of *SpeI* (Roche diagnostics, Manheim, Germany), and solved by electrophoresis with the same conditions used in the southern electrophoresis. Hybridized fragments with both probes in southern analysis, containing the sequences flanked to transposon were extracted from agarose gel and purified using the Qiaex II Handbook kit.

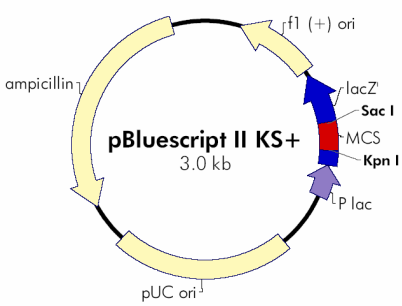

Figure 3.13. pBluescript II KS+ plasmid (Stratagene).

The plasmid pBluescript SK (+) was used for cloning DNA fragments. The pBS vector (2 µg) was digested at 37 ºC for 1 h with 1X of A buffer, 10 U of *AccI* and 20 U of *SpeI* (Roche diagnostics, Manheim, Germany) enzymes, in a final volume of 20 µl. Digested pBS was charged, solved and purified from 1.5% agarose gel with Qiaex II Handbook kit.

The quality and concentration of DNA preparations (insert and vector) were determined at 260 nm using a spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, DE) and by electrophoresis.

Several ligation reactions were performed in a final volume of 20 µl containing 1, 0.7, 0.5 µg of insert DNA, 0.2 µg of digested pBS vector, 1X of ligation buffer and 1 U of T4 DNA ligase (Roche diagnostics, Manheim, Germany). Ligation reactions were incubated overnight at 4 ºC. Two controls, a ligation reaction without DNA insert (named "pBS") and other with DNA insert extracted from parental strain (named "pBS-125") were added.

Preparation of competent cells

E.coli XL1-Blue strain was seeded on LB agar supplemented with tetracycline (Tc) (15 µg/ml) and incubated overnight at 37 ° C. A single colony was transferred in 5 ml of LB broth and incubated at 37 ° C overnight with shaking. A volume of 0.83-1 ml of culture was added to 250 ml SOB broth (annex I). Culture was incubated at 25 ºC with shaking during 4-5 h until an optical density of 0.6 at 600 nm was achieved. The culture was transferred into chilled autoclaved large centrifuge tubes and maintained for 10 min in ice cold. Tubes were centrifuged at 4000-5000 rpm for 10 min at 4 ºC. Cells must remain cold for the rest of the procedure. Supernatants were discarded and tubes were inverted maintained for 1 min. Cells were resuspended in 80 ml of ice cold TB buffer (annex I) by gentle mixing and suspensions held on ice for 10 min. Tubes

were centrifuged at 4000-5000 rpm for 10 min at 4 ºC and supernatants were discarded. Cells were resuspended in 18.6 ml of ice cold TB buffer. A volume of 1.4 ml of dimethyl sulfoxide (DMSO) (Sigma, Misouri, USA) was added to each tube and suspensions were held on ice for 10 min. Cells were aliquoted in volumes of 200 µl and tubes were placed immediately into a liquid nitrogen bath. Competent cell aliquots were stored at -80 º C.

Transformation procedure and selection

Tubes containing competent cells were maintained in ice cold until defrosting, about 30 min. The ligation product of 20 µl obtained above was added and gently mixed. Three controls based on *E. coli* cells transformed with pBS and pBS-125 plasmids and with water were included. The mix was kept on ice for 30 minutes. Tubes were heated at 42 °C for 30 s without agitation and transferred to ice for 1-2 minutes. A volume of 0.8 mL of SOC medium (annex I) was added to each tube and cells were incubated at 37°C for 45 min with vigorous shaking. Cells were spread on pre-warmed LB agar (37°C) supplemented with Amp (100 µg/ml), Sm (10 µg/ml) and Spc (25 µg/ml). Also controls were spread on plates containing LB agar supplemented with Amp (100 µg/ml). Plates were incubated overnight at 37 ºC. Recombinant clones resistant to Amp, Sm and Spc antibiotics were recovered and stored at - 80 ºC.

The presence of the vector carrying insert (transposon and target sequences) in recombinant clones was confirmed by digestion of plasmid with endonucleases enzymes that release the insert from pBS vector. Plasmid was extracted from overnight clone cultures using Plasmid extraction Wizard Plus SV Minipreps DNA purification system (Promega Corp., Tokyo, Japan). Digestion reaction was performed in a final volume of 20 µl containing 1X of L buffer, 10 U of *KpnI* (Roche diagnostics, Manheim, Germany), 10 U of *SacI* (Roche diagnostics, Manheim, Germany) and 200 ng of plasmid extraction at 37 ºC for 1h. The presence and the size of the insert and pBS was analyzed by electrophoresis following the procedure described above.

Sequencing

Plasmid material containing insertion transposon fragment from mutants was used in this section. The flanking regions corresponding to the minitransposon borders of mutants 40, 2002, 2126 and 4015 were sequenced using the the BigDye Terminator v3.0 Ready Reaction cycle sequencing kit (Applied Biosystems, California, USA). Sequencing was performed in Servei de Biologia Molecular dels Serveis Integrats de Recerca de la Universitat de Girona (Girona, Spain).

Table 3.4. Sequences of primers used to sequence region comprised between pBS plasmid and transposon for each mutant.

Primer	Sequenced mutant	Sequence (5'→3')	Reference
M13 fw	40, 2002, 2126, 4015	GTAAAACGACGGCCAGT	Stratagene
M13 rv	40, 2002, 2126, 4015	GGAAACAGCTATGACCATG	Stratagene
IPCRO fw	4015	CACTTGTGTATAAGAGTCAG	Ochman *et al.*, 1990
IPCRI rv	40, 2002, 2126, 4015	CAGATCTGATCAAGAGACAG	Ochman *et al.*, 1990
A fw	40	CAACATGTTGGGTTGCA	This work
C fw	2002, 4015	CTGAAATCCCGAAGGCAA	This work
F fw	2126	AACGGCGTTTTACGGAA	This work
D fw	2126	GTGGCGATTACGATGACA	This work
J fw	4015	GCATCACTGCTTAAGGGTGA	This work
M fw	4015	ACTTAAGCCTAGCTGGTCTCA	This work
N rv	4015	TATCATCAACCTGGTGTCCA	This work
K rv	4015	GCAACAGCCTGACTTCCTA	This work
P fw	4015	GATTCGTTCTGGCTCCA	This work

Each sequencing reaction was performed in a total volume of 10 µl containing 1 µM of primer, 3 µl of Terminator Ready Reaction Mix and 500 ng- 1 µg of plasmid product. In Table 3.4 are detailed all primers used in this section to sequence the region between pBS vector and transposon for each mutant. Primers developed in this work were designed using Primer

Express™ software (PE Applied Biosystems, MA, USA) from known sequences. DNA and translatable protein sequences were analyzed and related sequences in the GenBank database (2.0 version at NCBI) were identified using the BLAST protocols (Altschul *et al.*, 1990; Altschul *et al.*, 1997).

Transformation of *P. agglomerans* EPS125 by electroporation

With the aim to complement mutations produced in mutants, an efficient transformation method is essential. For this reason, a transformation protocol of *P. agglomerans* EPS125 by electroporation was developed varying several conditions in this section. The plasmid used for transformation was pBBR1MCS-3 (Kovach *et al.*, 1995) that contains a tetracycline resistance gene (Figure 3.14).

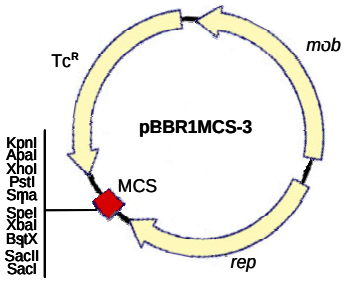

Figure 3.14. Scheme representing the construction of pBBR1MCS-3 plasmid.

A single colony was streak in LB agar and in 100 ml of LB broth. Two cultures were incubated overnight at 28-30 ºC and the liquid culture with shaking (125 rpm). The liquid culture was transferred in a chilled tube and maintained in ice cold during 15 min. Liquid culture was centrifuged for 10 min at 2000 g at 4 ºC and supernatant was discarded. The pellet was resuspended in 5 ml of ice cold 1 mM HEPES pH6.8 (H) and also in 5 ml of ice cold 1 mM HEPES pH6.8 and 10 % glycerol (HG). Bacterial mass of LB plate was collected in a chilled centrifuge tube containing 5 ml of ice cold H and HG wash buffers. A volume of 30 ml of H and HG solution, respectively, was added and tubes were mixed by pipetting. Tubes were centrifuged at 4000 rpm for 8 min at 4 ºC. On ice, supernatants were discarded and cell pellets washed twice with 25 ml of H and HG solution. Cell pellets were resuspended in 1 ml of H and HG solution and 200 µl were dispensed in chilled tubes. If competent cells are not used immediately, they are freeze in liquid nitrogen bath and stored at -80 °C. Different quantities (200, 600 and 1000 ng) of pBBR1MCS-3 plasmid (≤5 µl) were added to competent cells mixing by pipette. A negative control with water was included. Mix was transferred to a chilled BioRad Brand 0.2 cm gap electroporation cuvette (Bio-Rad, Hercules, CA) and maintained in ice cold. Strain EPS125 was electroporated in two conditions, at 2.45 kv/cm, 375 Ω and 25 µF and at 2.5 kv/cm, 200 Ω and 25 µF. After pulsing, 950 µL of SOC broth were added immediately to cuvette and mixed. The samples was removed from cuvette and placed in a new sterile tube. Cells were regained for 1 h at 30 ºC with shaking. Cells were spread on plates containing LB supplemented with Tc (20 µg/ml) and incubated overnight at 30 ºC. Transformed cells were recovered as colonies grown on LB+Tc medium.

4. Results

4.1. Phenotypic analysis of *Pantoea agglomerans* EPS125

4.1.1. Production of antifungal metabolites

PTFE filters containing *P. expansum* conidia were examined with optical microscope. Pathogen spores germinated in spent culture filtrates of EPS125, EPS484 and SBW25 grown in apple juice. These results show that in tested conditions *P. agglomerans* EPS125 does not produce antifungal substances or not in enough amounts to inhibit spore germination.

In addition, it was observed that strain EPS125 did not produced chitinases according to the *in vitro* assay after 6 days of incubation in plates containing chitin.

4.1.2. Competition for nutrients

The competition for nutrients was studied through two experimental designs according to the nutrient source. The first type of experiment (*in vitro*) used sterile 1% apple juice as nutrient source. The second type of experiment designated as *ex vivo* used disks of apple tissue (*ex vivo* A) and a piece of apple (*ex vivo* B) as nutrient source. In both types of experimental, strain EPS125 and the pathogen were kept in direct contact and also were separated physically by a semipermeable filter which permits medium nutrients and metabolite interchange.

Table 4.1 shows the results obtained in the three different assays. All spores of *P. expansum* germinated when EPS125 cells were separated from conidia by a filter. However, germination was prevented in the three assays by the addition of EPS125 in the same compartment.

Table 4.1. Germination of *P. expansum* spores in *in vitro* and *ex vivo* assays according to microscopy observations.

Experiment	Treatment	Insert	Well	Result
in vitro	Nontreated control	Fungal spores	-	+
	Direct contact	EPS125+ fungal spores	-	-
	Separated	Fungal spores	EPS125	+
ex vivo (A)	Nontreated control	Fungal spores	-	+
	Direct contact	EPS125+ fungal spores	-	-
	Separated	Fungal spores	EPS125	+
ex vivo (B)	Nontreated control	Fungal spores	-	+
	Direct contact	EPS125+ fungal spores	-	-
	Separated	Fungal spores	EPS125	+

4.1.3. Taxis towards spores of *P. expansum*

The ability of *P. agglomerans* EPS125 to move to spores and germ tubes of *P. expansum* was studied by means of *in vitro* assays. After the incubation period it was not observed taxis of EPS125 to either of two pathogen states.

4.1.4. Production of exopolysaccharides

FT-IR spectrometry was used to generate IR spectra from cultures of strain EPS125 grown in different media, osmoadaptation medium (SDM), LB broth and apple juice. Figure 4.1 shows IR spectra from 2000 to 400 cm $^{-1}$ obtained in the three conditions. The first IR spectrum corresponded to strain EPS125 grown in LB broth and contains bands at 1650 cm^{-1} (amide I); 1550 cm^{-1} (amide II); 1405 cm^{-1} (carboxylate ion) and 1090 cm^{-1} (P-O, C-OH stretching), which

are representative of a biofilm grown on a balanced medium (Nivens *et al.*, 1995). The amide I and II bands are present in all spectra indicating the presence of peptide and proteins in samples. When strain EPS125 was grown in SDM media and apple juice, spectral features distinguishing from LB spectrum were observed. The two FT-IR spectra contained, in addition to the spectral bands noted above, a peak at 1060 cm^{-1} (C-OH stretching of alginate) associated with the alginate polysaccharide.

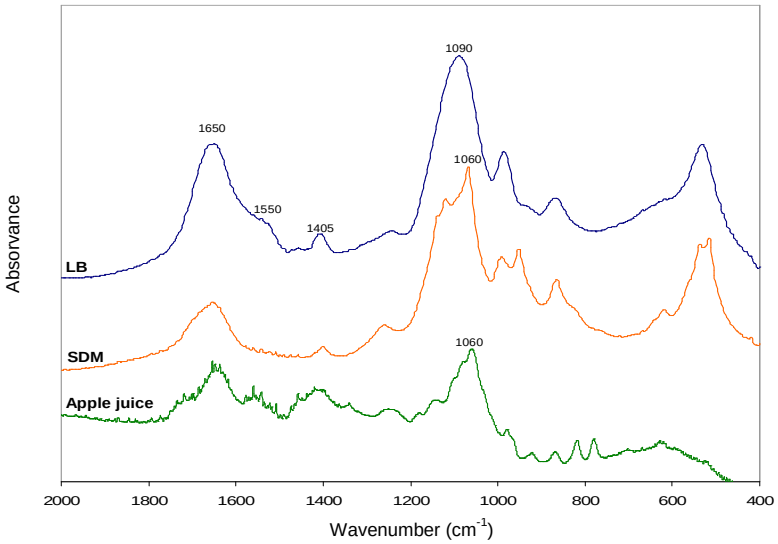

Figure 4.1. Spectra of EPS125 grown in LB broth (**LB**), SDM medium (**SDM**) and apple juice (**apple juice**). LB spectrum showing IR bands associated with cells: 1650 cm^{-1} (amide I); 1550 cm^{-1} (amide II); 1405 cm^{-1} (in part due to symmetric stretching of the carboxylate ions) and the 1100 to 1000 cm^{-1} region (P-O, C-OH stretching). SDM and apple juice spectra also show an IR band at 1060 cm^{-1} (C-OH stretching) associated with the alginate.

4.1.5. Analysis of *P. agglomerans* EPS125/*P. expansum* interaction by SEM

Interaction of the strain EPS125 and the pathogen was observed by means of SEM. Filters of inserts of Millicell® tissue culture plates (*in vitro* assay) and wounds produced on apple fruit and treated with pathogen and BCA (*ex vivo* assay) were examined.

The micrographs of filters containing *P. expansum* spores incubated for different periods showed the germination process from conidia of 5-6 μm of diameter (Figure 4.2 A), formation of germ tubes (Figure 4.2 B and C), and development of mycelium (Figure 4.2 D).

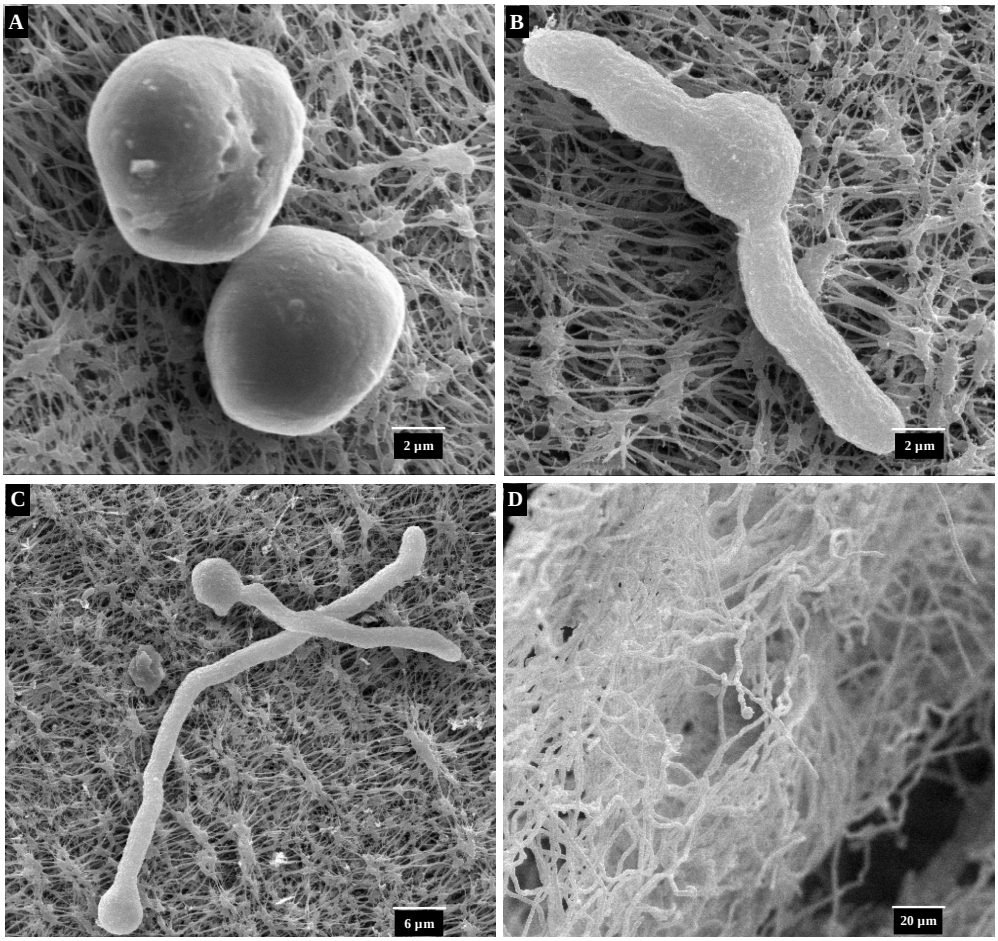

Figure 4.2. SEM micrographs of filters containing pathogen spores in different phases of development. **A:** 1 h. **B and C:** 4h. **D:** 13 h of incubation.

When *P. expansum* spores were in direct contact with *P. agglomerans* EPS125 cells, rod cells of 1 µm length and 0.5 µm diameter, BCA cells showed pilus-like structures (PLS) that interconnected antagonist cells forming cellular aggregates or 'symplasmata' (Figure 4.3 A) and facilitating the bacterial adhesion to the surface of fungal spores in the *in vitro* assay (Figure 4.3 B and C).

In the *ex vivo* assay, wounds produced on apple fruit (Figure 4.3 D) were inoculated with *P. expansum* spores and incubated 13 h allowing the germination of conidia, the growth of mycelium and the generation of new spores of the pathogen (Figure 4.3 E and F; and Figure 4.4 A and B).

Micrographs of the apple wound treated with strain EPS125 showed cellular aggregations adhered to the surface apple wound with pili (Figure 4.5 A, B, C and D). When apple wounds were treated with antagonist strain and inoculated with spores of *P. expansum*, any pathogen spore germinated after 13 h of incubation. Figure 4.6 (A and B) shows as antagonist cells covered partial or totally conidia of *P. expansum* avoiding the germination and infection process of pathogen.

Finally, the interaction of EPS125 cells with germ tubes of the pathogen was observed when the apple wound was inoculated with *P. expansum* spores and treated 24 h afterwards with antagonist strain (Figure 4.7 A and B).

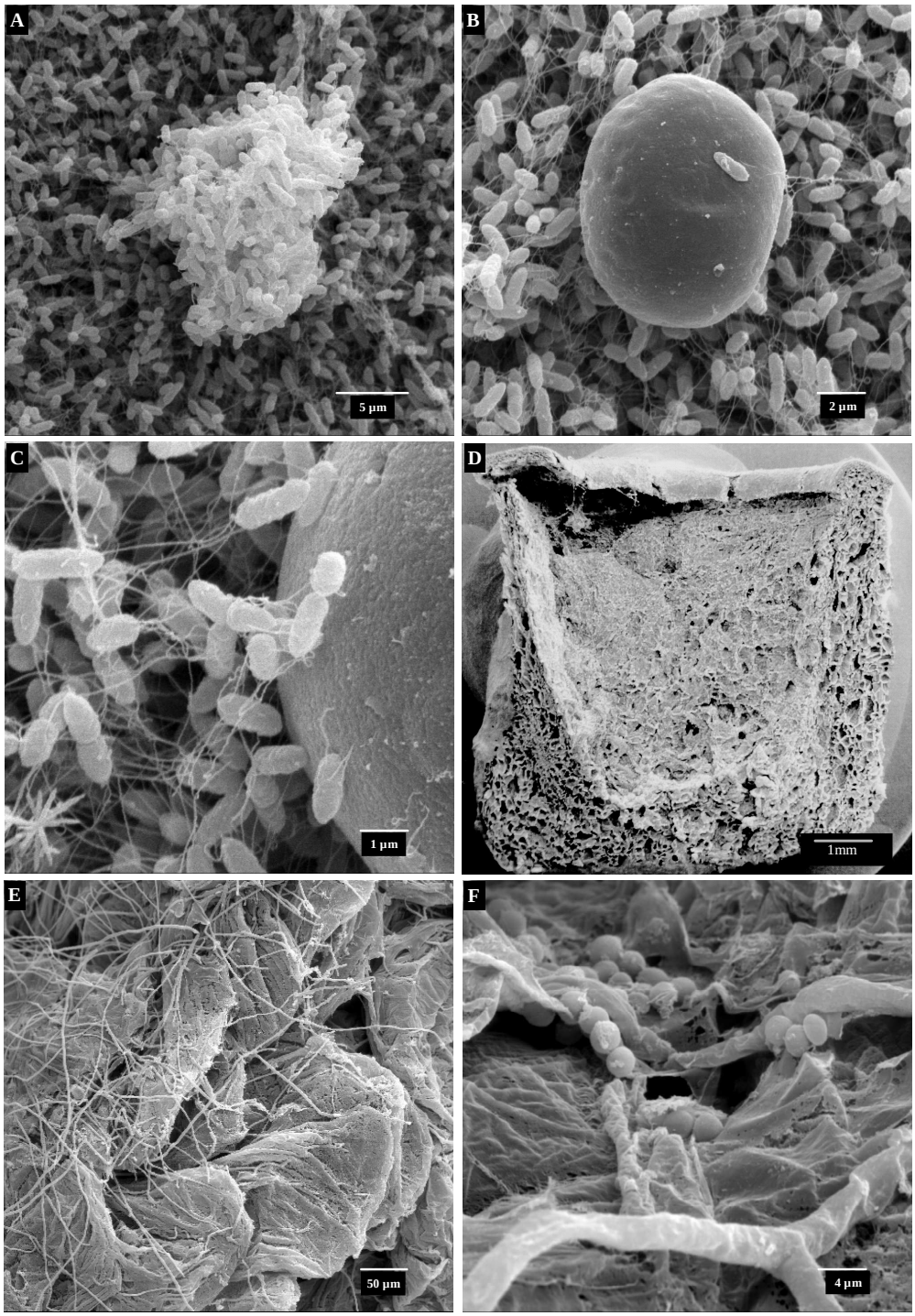

Figure 4.3. SEM micrographs of filters containing *P. expansum* spores and *P. agglomerans* EPS125 cells after 13 h of incubation (A, B and C) and surface of apple wounds inoculated with pathogen spores (D, E and F). **A:** Bacterial aggregate or 'symplasmata'. **B:** Pathogen spores in contact with antagonist cells. **C:** Higher magnification of bacteria cells with PLS linking cells and adhering to conidia surface. **D:** Longitudinal section of a wound produced on apple fruit. **E:** Pathogen mycelium invading the apple tissue after 13 h of incubation. **F:** High magnification of mycelium and spores formed by *P. expansum* on apple surface after 13 h of incubation.

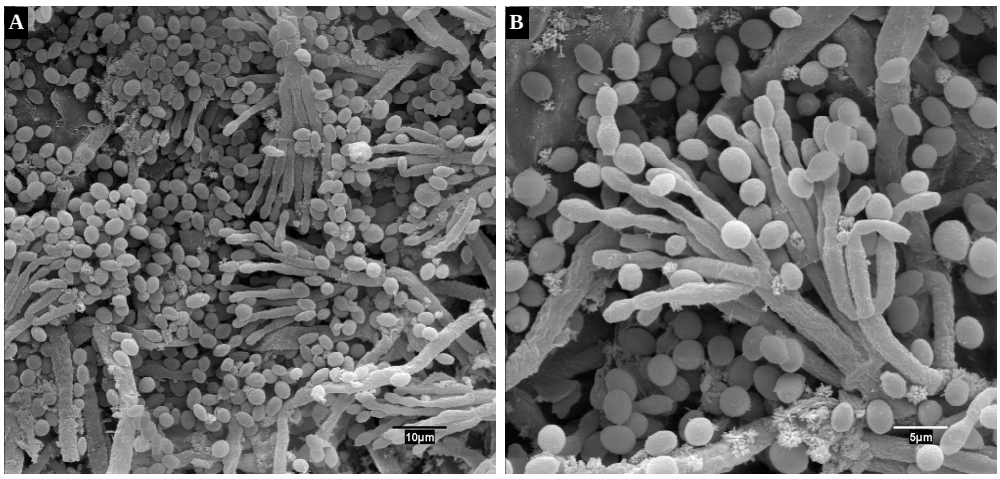

Figure 4.4. SEM micrographs of the surface of apple wounds inoculated with *P. expansum* spores and incubated for 13 h. **A and B:** Conidia and conidiophores produced on infected wound surface.

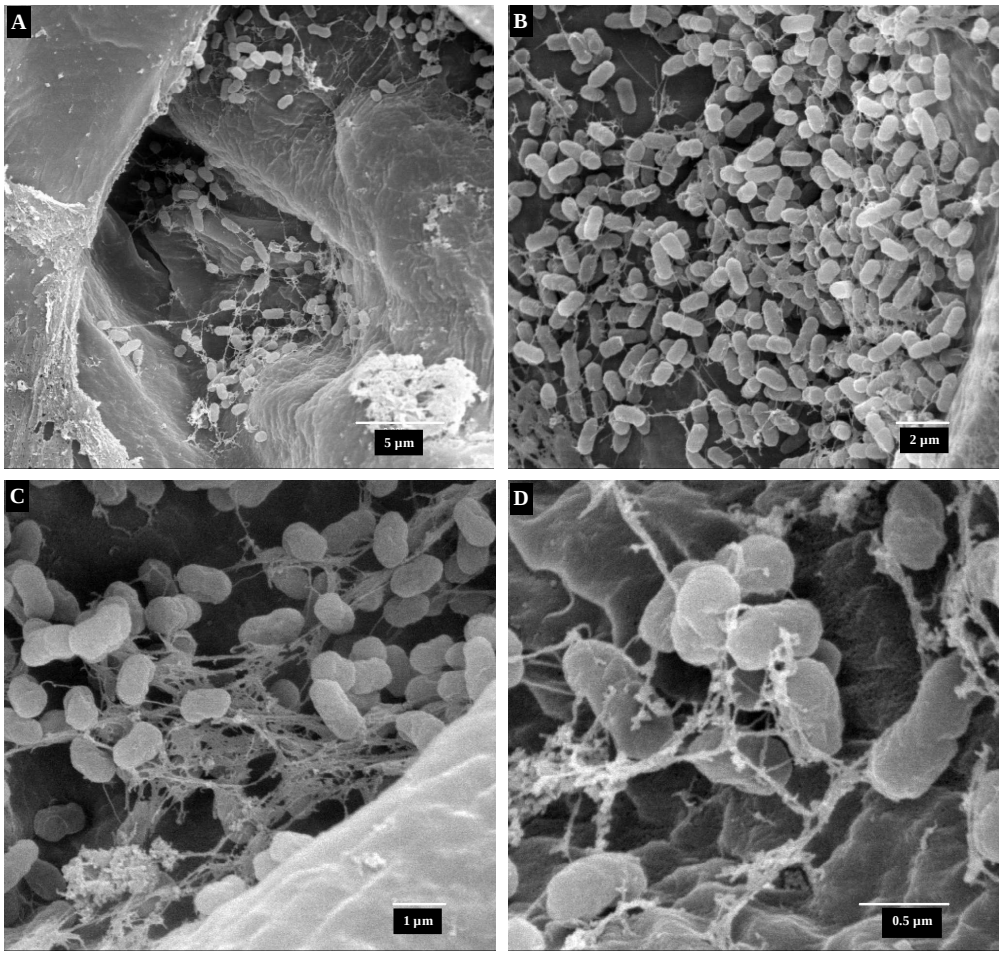

Figure 4.5. SEM micrographs of the surface of apple wounds treated with strain EPS125 and incubated for 13 h. **A and B:** Bacterial cells adhered to apple tissue with PLS. **C and D:** Details of bacteria agglomerated and adhered to apple surface.

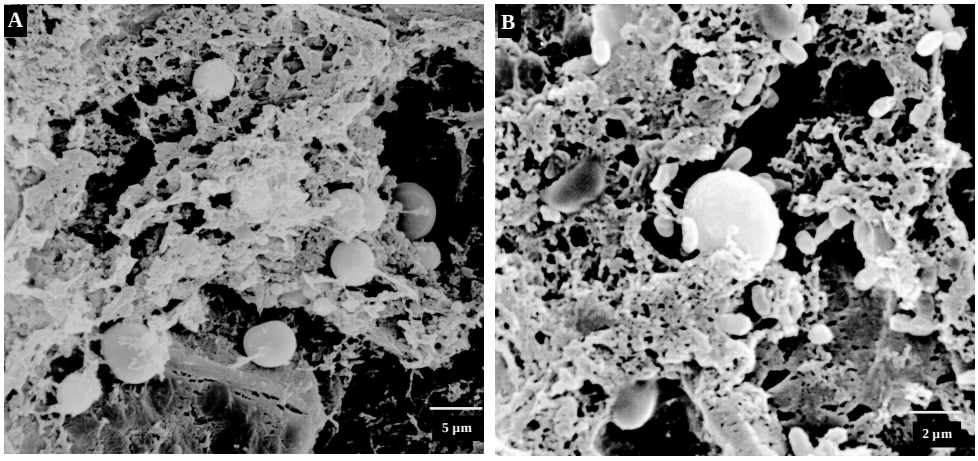

Figure 4.6. SEM micrographs of the surface of apple wounds treated with strain EPS125, inoculated with pathogen spores and incubated for 13 h. **A and B:** Pathogen spores surrounded and inhibited by EPS125.

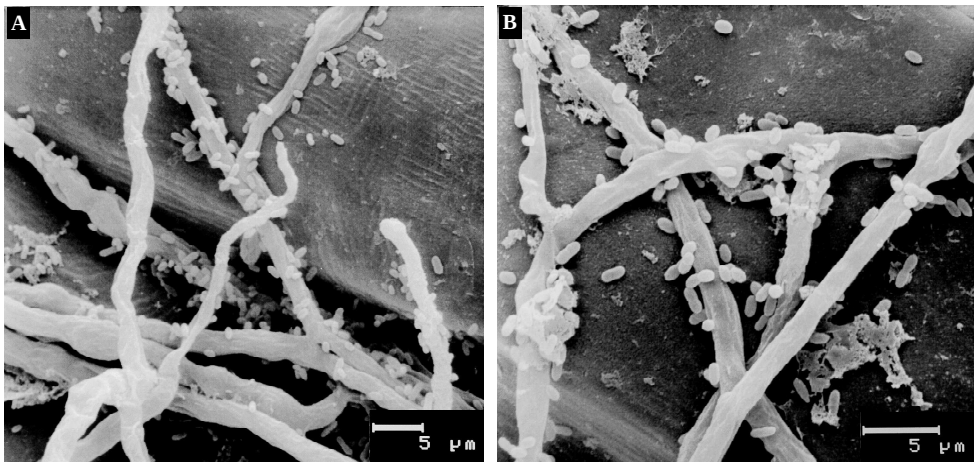

Figure 4.7. SEM micrographs of the surface of apple wounds after 24h from treatment with strain EPS125 and inoculated with pathogen spores. **A and B:** EPS125 cells adsorbed to germ tubes of *P. expansum*. Notice that several EPS125 cells adhered to germ tubes are under cell division.

It has to take into account during the SEM fixation process of samples that bacterial and fungal cells attached to apple tissue may be lost due to the continuous change of solutions.

4.1.6. Analysis of *P. agglomerans* EPS125/*P. expansum* interaction by TEM

Cultures of *P. agglomerans* EPS125 grown in LB broth and apple juice were observed by means of TEM. Ultrathin sections were stained with ruthenium red to detect saccharic components on the bacterial surface.

Transmission electron micrographs of a culture of EPS125 grown with LB showed PLS linking cells (Figure 4.8 A and B). PLS also were observed when strain EPS125 was grown in apple juice (Figure 4.8 C). In addition, ruthenium red-stained transmission electron micrographs of strain EPS125 grown in apple juice showed electrodens saccharic granules around surface of cells (Figure 4.8 D) that, in an advanced phase, fused forming a layer that grouped cells (Figure 4.9 A, B, C and D). These cell aggregates or symplasmata were not detected when strain EPS125 was grown in LB broth. The electrodens granules and layers surrounding cell aggregates were

not visualized in TEM micrographs of ultrathin sections of EPS125 grown in apple juice without stain with ruthenium red.

Negative staining micrographs of strain EPS125 grown in apple juice showed a large amount of long PLS interconnecting cells (Figure 4.10 A and B).

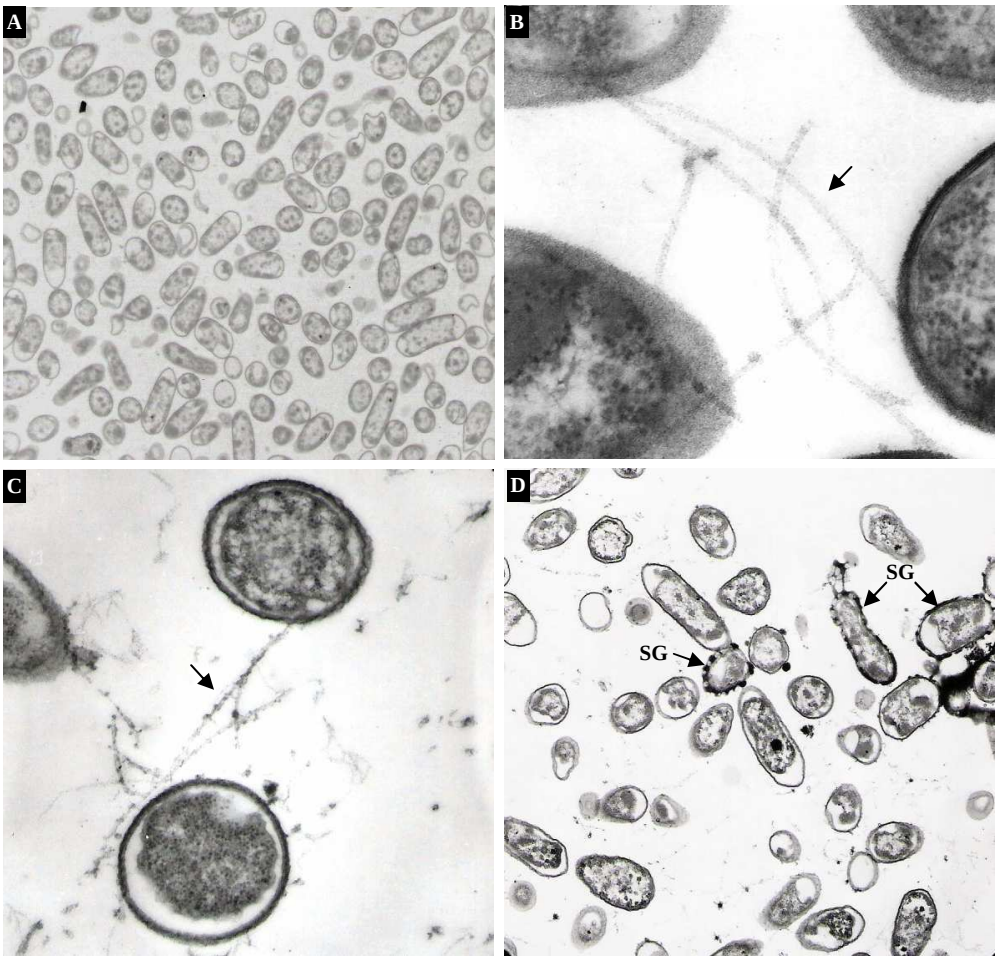

Figure 4.8. TEM micrographs of ultrathin sections stained with ruthenium red of EPS125 cells grown in LB broth. **A:** General view of EPS125 cells (6,300X). **B:** Cells interconnected with PLS (40,000X; 63,000X; and 80,000X). **C:** PLS connecting cells of EPS125 (40,000X). **D:** EPS125 cells surrounded of electrodens granules (10,000X). Black arrows: PLS. SG (arrows): Saccharic granules.

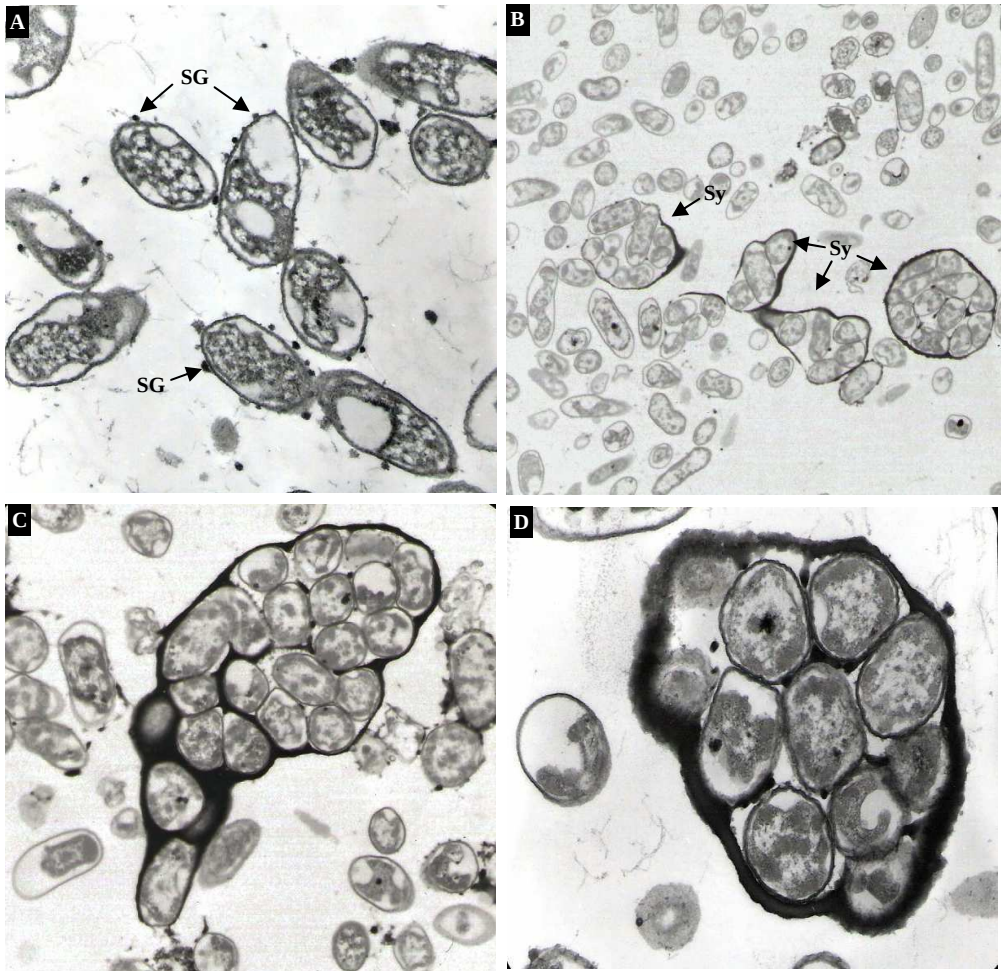

Figure 4.9. TEM micrographs of ultrathin sections stained with ruthenium red of EPS125 cells grown in apple juice. **A:** Cells surrounded of electrondens granules (20,000X). **B:** Cell aggregates or symplasmata surrounded of a saccharic layer stained with ruthenium red (6,300X). **C and D:** High magnification of symplasmata stained with ruthenium red (12,500X; 25,000X). SG (arrows): Saccharic granules. Sy (arrows): Symplasmata.

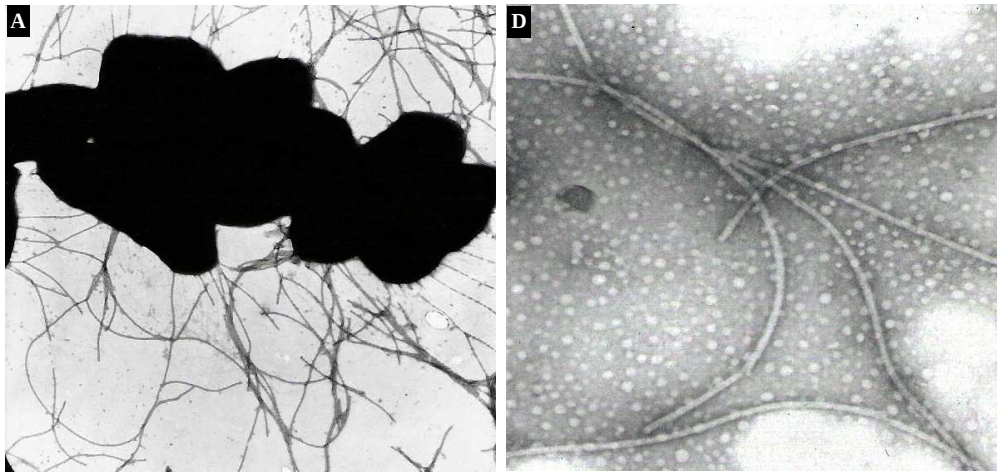

Figure 4.10. Negative staining micrographs of EPS125 cells grown in apple juice. **A:** Large amount of long PLS (40,000X). **B:** High magnification of PLS (100,000X).

4.2. Mutagenesis with transposons and mutant characterization

To determine the mechanism of action used by *P. agglomerans* EPS125 in the biocontrol of *P. expansum* on apple fruit, the holistic technique of random transposon mutagenesis was used. Minitransposon mTn5SS*gusA40* containing a promoterless reporter gene GUS and Sm/Spc genes was introduced into strain EPS125 by conjugation with *Escherichia coli* S17.1-λpir (pCAM140). Based on antibiotic markers selection (resistance to Sm and Spc), a collection of 4032 auxotrophic mutants was obtained from a mating experiment.

4.2.1. Selection of mutants

To select mutants defective in biocontrol of *P. expansum* on apple fruit, a bioassay with appropriate bacterial and pathogen concentrations was necessary. For this purpose, a dose-response assay of strain EPS125 with the pathogen *P. expansum* on apple fruit was performed (see '4.1.6. Dose-response assays. Chapter I' section). According to results showed in dose-response assays, it was decided to treat apples with 10^9 cfu/ml of bacterial suspension and $5x10^4$ conidia/ml of the pathogen suspension because using these concentrations EPS125 achieved 100% of suppression of disease.

The 4032 mutants were submitted to a screening process to select those mutants that have lost their biocontrol activity against *P. expansum* on apple fruit (Figure 4.11). From the first screening 63 mutants (1.56%) were selected being totally defective in biocontrol capacity (0 % biocontrol). Of these, 56 mutants maintained the altered phenotype in the second screening. From this group, 12 mutants were selected according to the stability of biocontrol results and were submitted to another additional assay. All 12 mutants continued being defective in biocontrol in the third screening. Finally, m40, m439, m622, m1210, m2002, m2126 and m4015 mutants were chosen to perform phenotypic and genotypic studies.

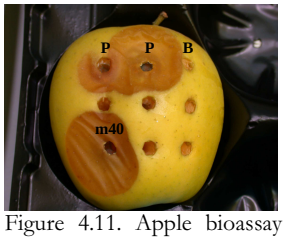

Figure 4.11. Apple bioassay showing the mutant m40 defective in biocontrol. **P:** Pathogen positive control; **B:** EPS125 control; **m40:** mutant m40.

4.2.2. Phenotypic studies of mutants

All the mutants selected for their impaired biocontrol activity (m40, m439, m622, m1210, m2002, m2126 and m4015) were studied in relation to colony morphology, growth potential in LB culture broth, growth on apple fruit, induction of GUS gene, quorum sensing, production of exopolysaccharides and interaction with pathogen cells.

Colony morphology

All 7 mutants showed the colony morphology of the parental strain EPS125 when were grown on ABM minimal medium. They shared the same colonial shape and mucus level and showed a yellow-orange pigmentation.

Growth potential in culture broth

The growth curves of mutants (m40, m439, m622, m1210, m2002, m2126 and m4015) obtained in LB broth were compared with the growth curve of *P. agglomerans* EPS125 using Bioscreen method. Tukey's mean separation test (P=0.05) performed on the area under the growth curve and the generation time showed no significant differences between mutants and the parental strain (Table 4.2). However, in relation with generation time significant differences were observed between m40 and m1210; m622 and m2002; m1210 and m2002; and m1210 and

m2126 mutants. Nevertheless, these results were highly variable due to difficulties in establishing the beginning point chosen from exponential growth curve.

Table 4.2. Area under the growth curve (agc) and generation time (gt) of mutants and the parental strain EPS125.

Mutant/wt	gt (min)[a]		agc [a]	
EPS125	60.77	abc	37.66	a
m40	66.95	ab	37,80	a
m439	60.91	abc	35,39	a
m622	54.66	bc	35,66	a
m1210	52.48	c	36,35	a
m2002	67.49	a	37,89	a
m2126	66.93	ab	36,50	a
m4015	58.73	abc	35,97	a

[a] gt and agc values are the mean of three replicates
Means within the same column followed by the same letter do not differ sifnificantly (P=0.05) according to Tukey's mean separation test. wt, wild-type strain.

Growth on apple fruit

Strain EPS125Rif and m40, m439, m622, m1210, m2002, m2126 and m4015 mutants colonized and grew rapidly in apple fruit wounds at 13 and 23 ºC. The population levels of EPS125 Rif and mutants in wounds, immediately after application, were approximately $5.4x10^5$ cfu/wound and increased within 21.5 h to $6.2x10^6$ to 10^7 cfu per wound at 13 ºC and to $1.2x10^7$ to $5x10^7$ cfu per wound at 23 ºC (Figure 4.12). Population levels of strain EPS125 Rif and mutants were similar and remained stable during the following 3 days of incubation at both temperatures assayed. Analysis of covariance showed no significant differences between strain EPS125 and mutants in relation to the number of cfu/wound at 13 ºC (P=0.98) and at 23 ºC (P=0.99).

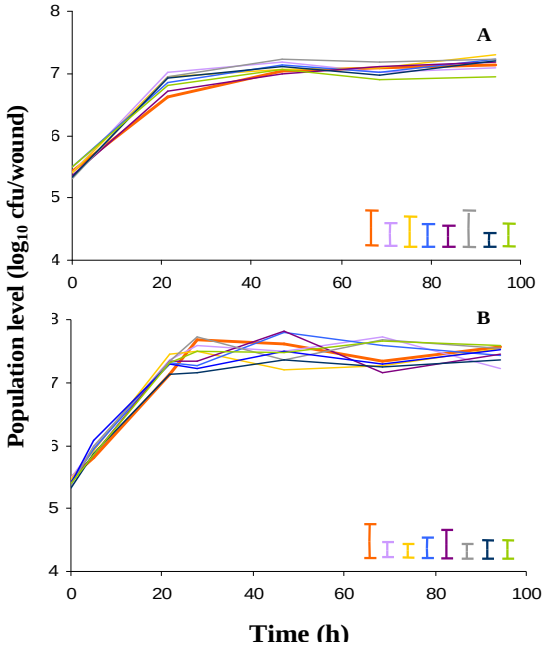

Figure. 4.12. Growth of parental strain EPS125 and mutants in apple wounds at 13 ºC (**A**) and 23 ºC (**B**). EPS125 (■), m40 (■), m439 (■), m622 (■), m1210 (■), m2002 (■), m2126 (■) and m4015 (■). Values are the mean of three replicates. Error bars indicate the confidence interval for the mean.

Induction of GUS gene

The minitransposon mTn5SS*gusA40* containing the promoterless reporter gene GUS allowed to study the induction of up-regulated disrupted genes of mutants in two *in vitro* conditions (LB broth and apple juice) (Figure 4.13) (Table 4.3). Strain EPS125, the negative control, did not have GUS activity in any condition tested. The mutants m439, m622 and m2002 did not show induction of reporter gene GUS in LB media nor apple juice. The expression levels of gene GUS in mutants m40 and m1210 was stronger in apple juice than in LB broth. The mutant 4015 only showed GUS activity in one of three replicates performed in apple juice. Finally, the mutant m2126 showed a variable expression of reporter gene with a more intense signal in LB broth.

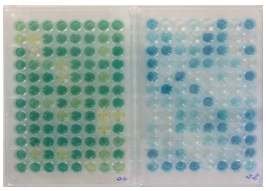

Figure 4.13. Expression of the reporter gene GUS of mutants grown in LB broth (left) and in apple juice (right).

Table 4.3. GUS gene expression of strain EPS125 and mutants when grown in LB broth or apple juice supplemented with X-GlcA.

Mutant/wt	LB			Apple juice		
EPS125	-	-	-	-	-	-
m40	(+)	(+)	(+)	+	+	+
m439	-	-	-	-	-	-
m622	-	-	-	-	-	-
m1210	-	(+)	+	+	+	+
m2002	-	-	-	-	-	-
m2126	-	(+)	+	-	-	(+)
m4015	-	-	-	-	+	-

The expression level of paranitrophenyl-β-D-glucuronide (pNPG) was rated using visual colour intensity: -, suspension colour similar to negative control; (+), suspension colour bluish (juice) or greenish (LB); and +, blue (juice) or green (LB) suspension colour. wt:, wild-type. Results obtained from the three repetitions.

Quorum sensing

The production of N-acyl homoserine lactone (AHL) signal molecules was studied using two bacterial sensor systems based on the macroscopic detection of a violet pigment produced by *Chromobacterium violaceum* and the GFP by *E. coli* JB525. The positive control *Serratia liquefaciens*, strain EPS125 and all mutants tested showed AHL signal in both systems.

Production of exopolysaccharides

FT-IR spectrometry has been used to compare absorption patterns of EPS125 with selected mutants when they were grown in LB broth and apple juice. Figure 4.14 shows IR spectra from 2000 to 400 cm $^{-1}$ of strain EPS125 and mutants m2126 and m4015 grown in LB broth. All IR spectra contains bands at 1650 cm^{-1} (amide I); 1550 cm^{-1} (amide II); 1405 cm^{-1} (carboxylate ion) and 1090 cm^{-1} (P-O, C-OH stretching). Figure 4.15 shows the IR spectra of strain EPS125 and mutants m40, m439, m622, m1210, m2002, m2126 and m4015 grown in apple juice. Mutants maintained the same FT-IR spectrum of strain EPS125, which contains the spectral bands noted above and a characteristic and well defined band at 1060 cm^{-1} (C-OH stretching of alginate) corresponding to alginate polysaccharide.

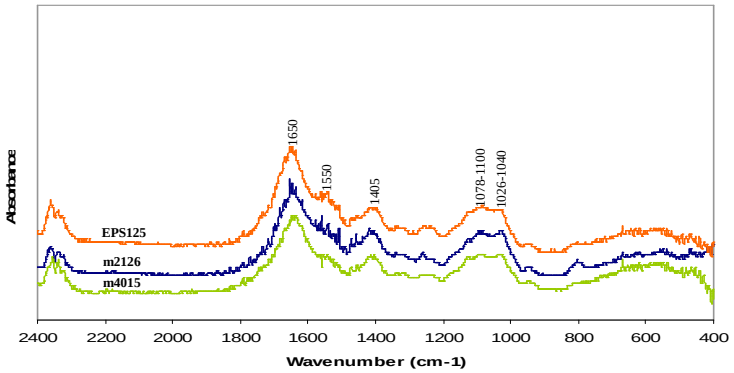

Figure 4.14. Spectra of EPS125 and mutants m2126 and m4015 grown in LB broth. All spectra show IR bands associated with cells: 1650 cm^{-1} (amide I); 1550 cm^{-1} (amide II), 1405 cm^{-1} (in part due to symmetric stretching of the carboxylate ions) and the 1100 to 1000 cm^{-1} region (P-O, C-OH stretching).

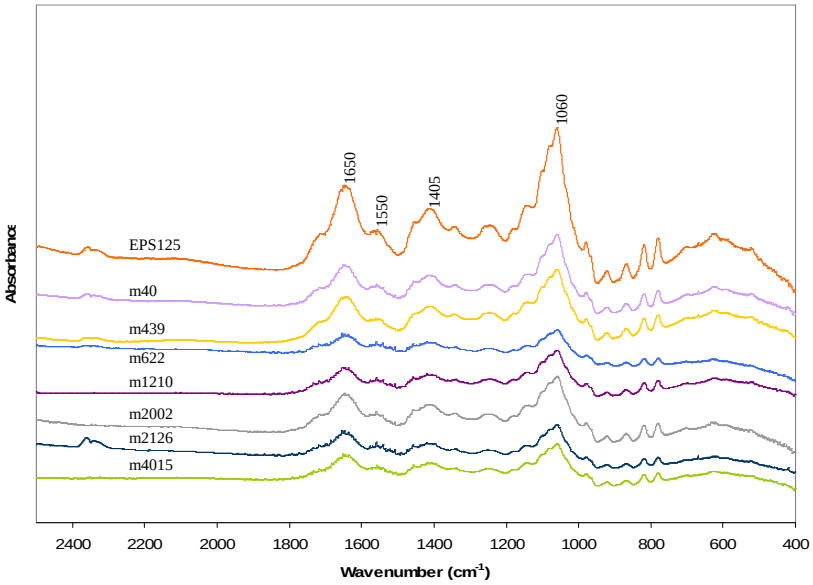

Figure 4.15. Spectra of EPS125 and mutants m40, m439, m622, m1210, m2002, m2126 and m4015 grown in apple juice. All spectra show IR bands associated with cells: 1650 cm^{-1} (amide I); 1550 cm^{-1} (amide II) and 1405 cm^{-1} (in part due to symmetric stretching of the carboxylate ions); and an IR band associated with alginate: 1060 cm^{-1} (C-OH stretching).

Biocontrol agent mutants and pathogen interaction studied by SEM

Interaction between m40, m439, m622, m1210, m2002, m2126 and m4015 mutant cells and spores of *P. expansum* was examined by means of SEM in comparison with parental strain in *in vitro* and *ex vivo* assays.

All spores of *P. expansum* germinated after 24 h of incubation period with apple juice (Figure 4.16 A and B), but when they were incubated together with EPS125 cells, conidia did not germinate (Figure 4.17 B). SEM micrographs of EPS125 cells incubated in direct contact with pathogen spores also show large cellular aggregates (Figure 4.17 A, C and D) and abundant PLS interconnecting cells of strain EPS125 (Figure 4.17 B, C and D; Figure 4.18 A and B) and surrounding pathogen spores (Figure 4.17 B).

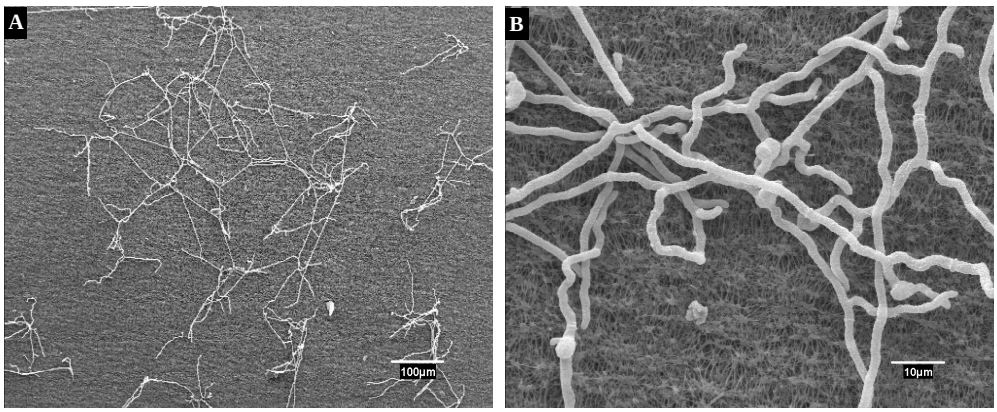

Figure 4.16. SEM micrographs of filters containing *P. expansum* spores. **A and B:** Germinative tubes formed by *P. expansum* after 24 h of incubation in apple juice.

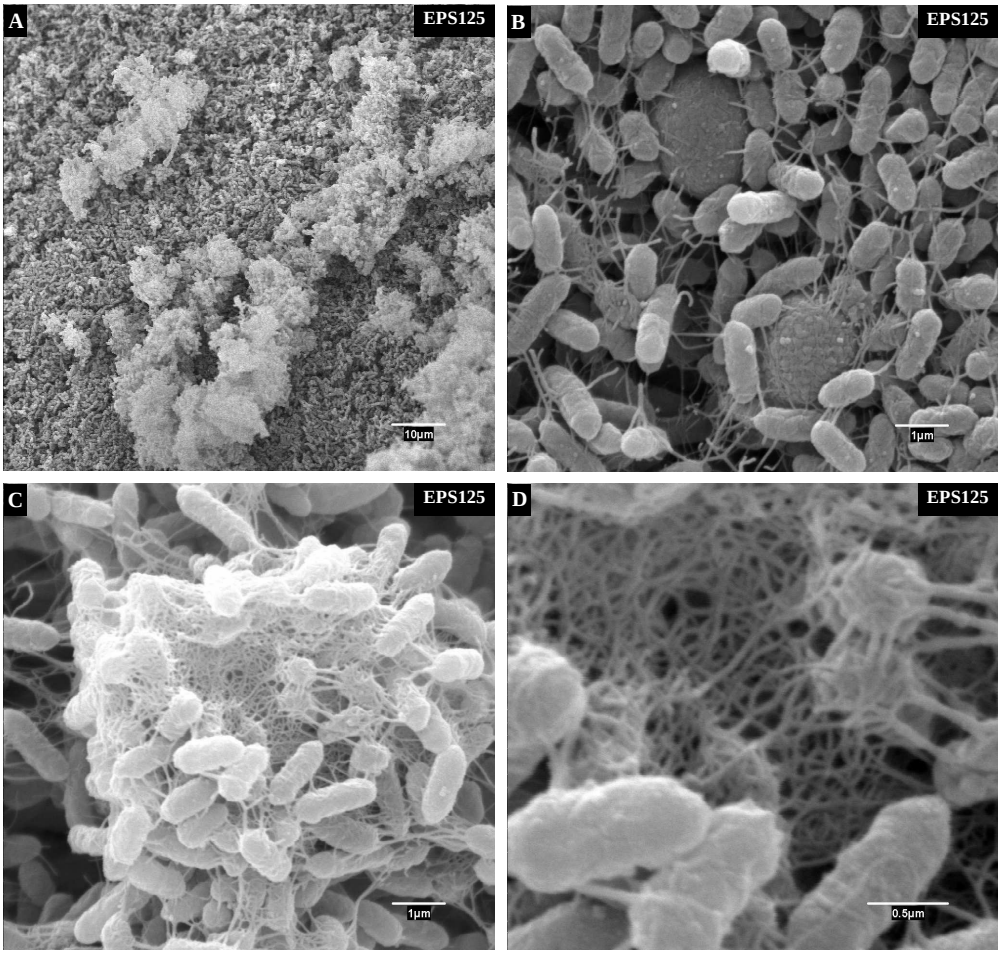

Figure 4.17. SEM micrographs of filters containing *P. expansum* spores and *P. agglomerans* EPS125 cells. **A and C:** Cellular aggregates of strain EPS125. **B:** Antagonist cells adhered to pathogen spores. **D:** Higher magnification of PLS linking cells.

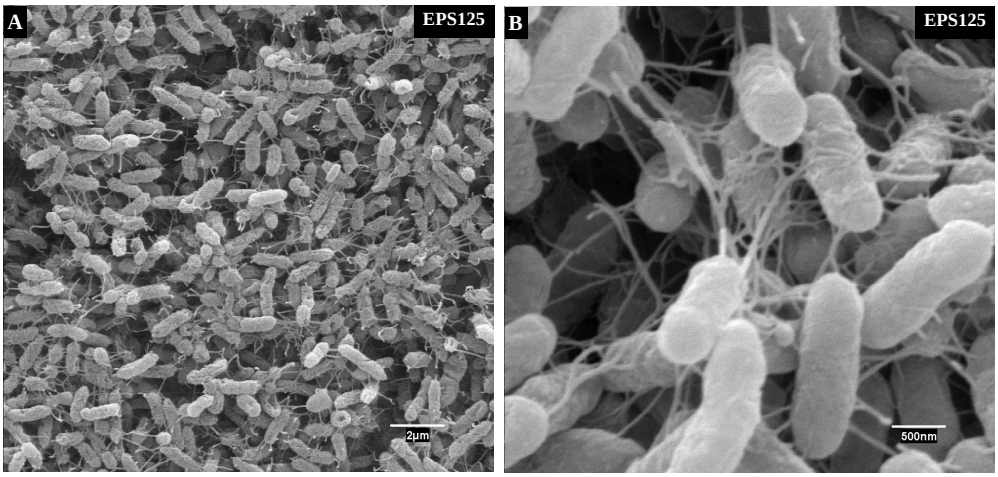

Figure 4.18. SEM micrographs of filters containing *P. expansum* spores and *P. agglomerans* EPS125 cells. **A:** Cells of strain EPS125 linked by PLS. **B:** Higher magnification of PLS linking cells.

The mutants m40, m439, m622, m1210 and m2002 showed a phenotype similar to parental strain EPS125 after 24 h of incubation with spores of *P. expansum* using apple juice as nutrient source. Conidia still have not germinated and bacterial cells were agglomerated and interconnected by PLS (Figure 4.19).

As it has been described above with other mutants, pathogen spores did not germinate yet when were incubated together with m2126 mutant for 24 h, however the m2126 cells were phenotypically different than EPS125 cells (Figure 4.20 A, B and C). SEM micrographs at low magnification show that m2126 did not form the large cellular aggregates formed by EPS125 and mutant cells were maintained on a same plane (Figure 4.20 A). In addition, high magnification SEM micrographs showed m2126 cells were different to parental strain according the amount of PLS and the kind of cellular interactions (Figure 4.20 B and C). There were few or none PLS linking cells and mutant cells were closely linked to filter surface and sometimes were covered by an unidentified substance (Figure 4.20 C).

Cells of mutant m4015 also showed an altered phenotype in comparison with strain EPS125 when were incubated overnight with *P. expansum*. Low magnification micrographs show that pathogen spores did not germinate and bacterial cells did not form the characteristic cellular agglomerates of strain EPS125 (Figure 4.20 D). In high magnification SEM micrographs showed few PLS linking cells and cells were covered by an unidentified amorphous substance grouping cells (Figure 4.20 E and F).

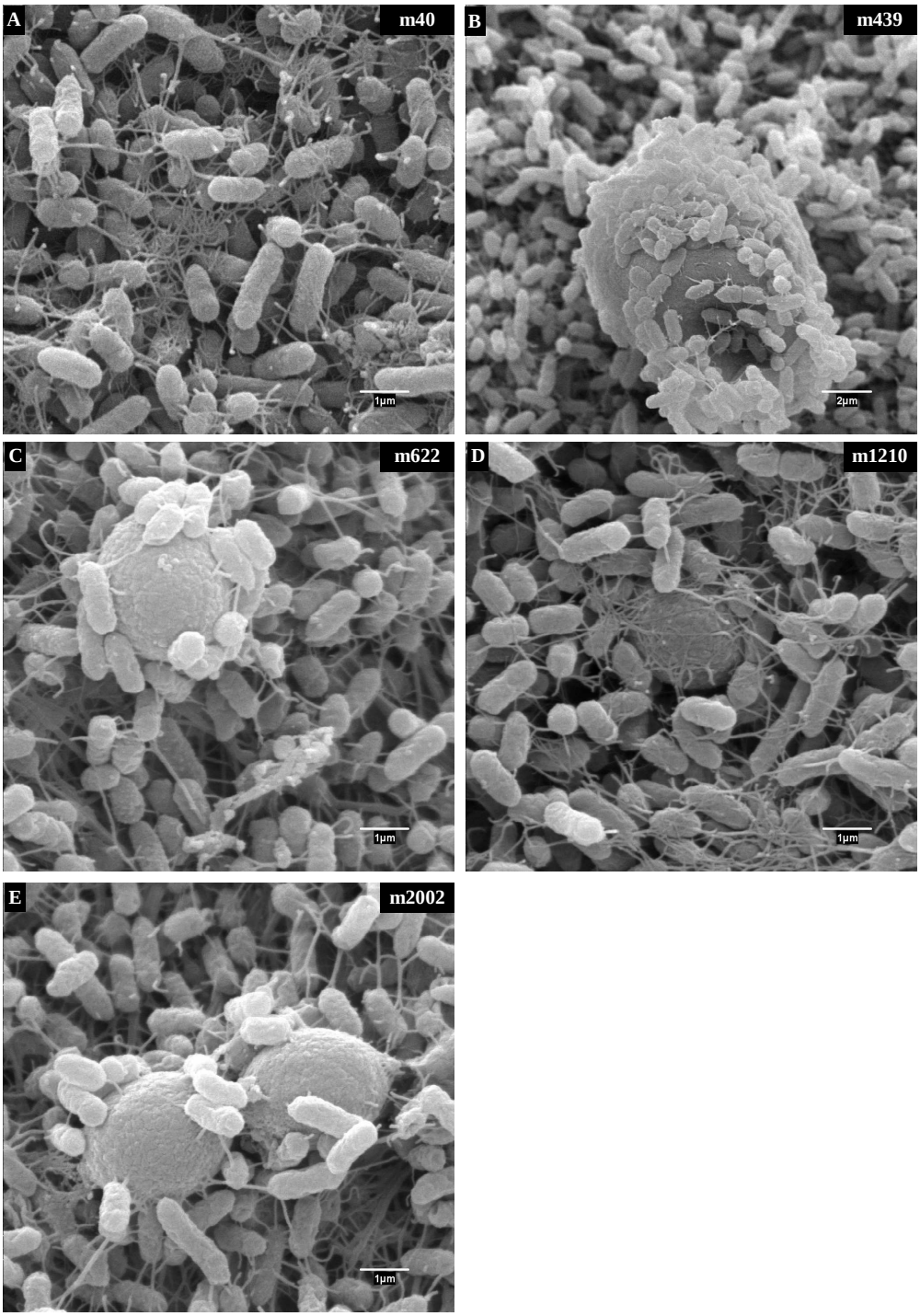

Figure 4.19. SEM micrographs of filters containing *P. expansum* spores and mutant cells. **A:** PLS linking m40 cells. **B:** m439 cells surrounding pathogen spores with PLS. **C:** m622 cells surrounding pathogen spores with PLS. **D:** m1210 cells surrounding pathogen spores with PLS. **E:** m2002 cells surrounding pathogen spores with PLS.

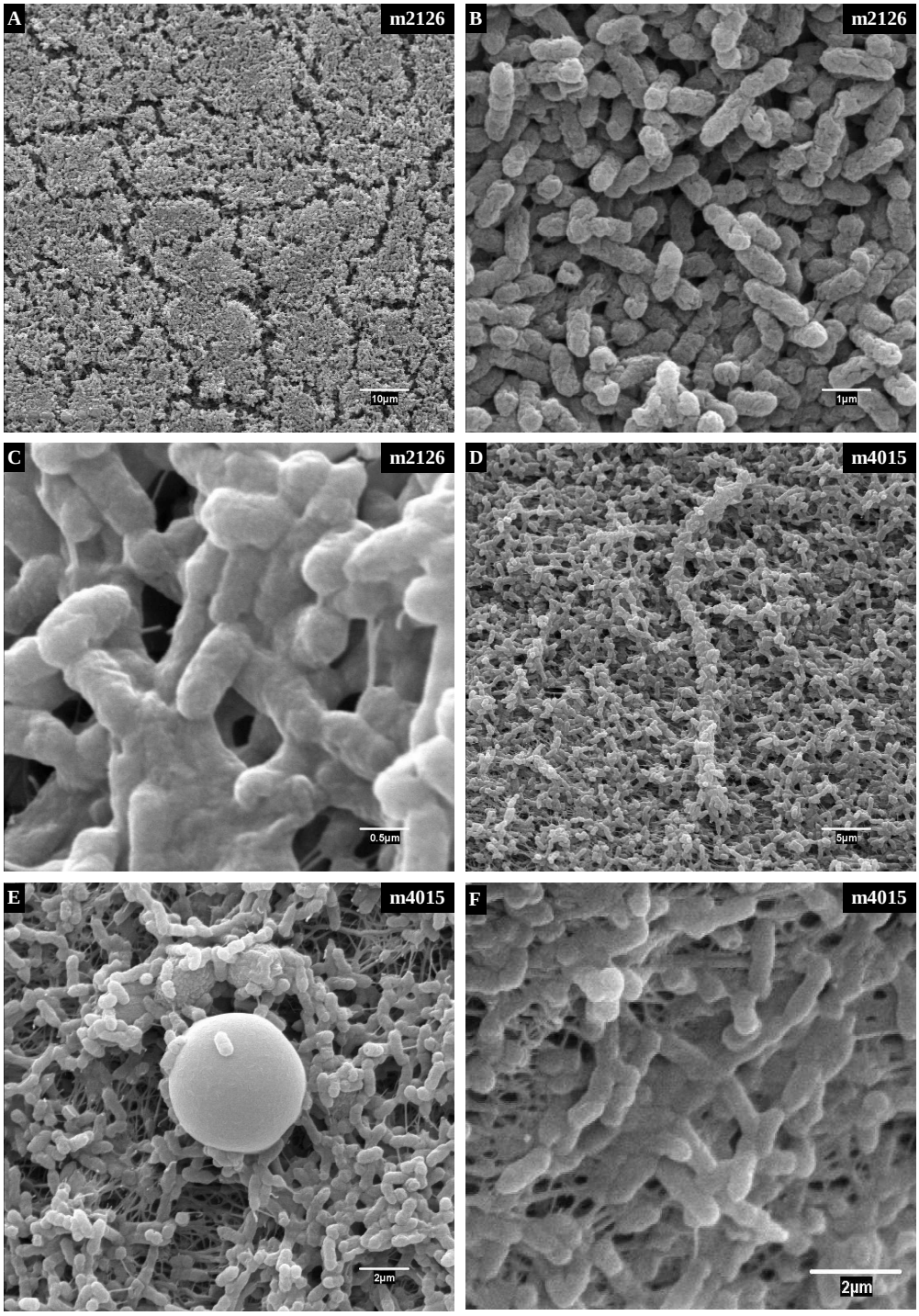

Figure 4.20. SEM micrographs of filters containing *P. expansum* spores and cells of m2126 and m4015 mutants. **A:** Low magnification of cells of mutant m2126 deposited on filter surface. **B:** High magnification of m2126 cells without PLS. **C:** High magnification of m2126 bacterial cells linked to filter surface and covered by an unidentified substance. **D:** Cells of mutant m4015 aggregated by an unidentified substance. Some PLS interconnecting cells are observed. **E:** Pathogen spore in contact with cells of mutant m4015 agglomerated and linked by PLS. **F:** High magnification of m4015 cells linked to filter surface and covered by an unidentified substance.

Apple wounds treated with water, strain EPS125 and all seven mutants and inoculated with pathogen spore suspension were incubated for 5 days at 23 ºC.

Micrographs of apple wounds inoculated only with *P. expansum* spores show that all conidia germinated and a dense mycelium with conidiophores was formed (Figure 4.21). However, the pathogen development in apple wound was inhibited when it was incubated together with cells of strain EPS125. Figure 4.22 illustrates how EPS125 cells expanded on apple wound surface forming cellular agglomerations and, in some cases, surrounding pathogen spores inhibiting the germination process. The PLS formed by strain EPS125 were observed more difficult in apple wounds than in SEM micrographs performed on filters. It may be due to the need of more manipulation of apple samples during the SEM processing.

When pathogen spores were incubated with mutant cells of m40, m439, m622, m1210, m2002, m2126 and m4015 for 5 days, all conidia germinated forming a mycelium with conidiophores covering the wound surface. Bacteria cells of the seven mutants showed a phenotype similar to parental strain (Figure 4.23, 4.24, 4.25, 4.26) with cellular agglomerates surrounded the wound surface and spores and germ tubes of the pathogen. PLS of mutants interconnecting cells were not observed clearly.

SEM micrographs of apple wounds treated with m40 and m622, and inoculated with *P. expansum* showed a large amount of star-shaped salt precipitations on the surface of the wound, bacteria and mycelium of the pathogen making difficult the observation of the m40-pathogen-apple interaction.

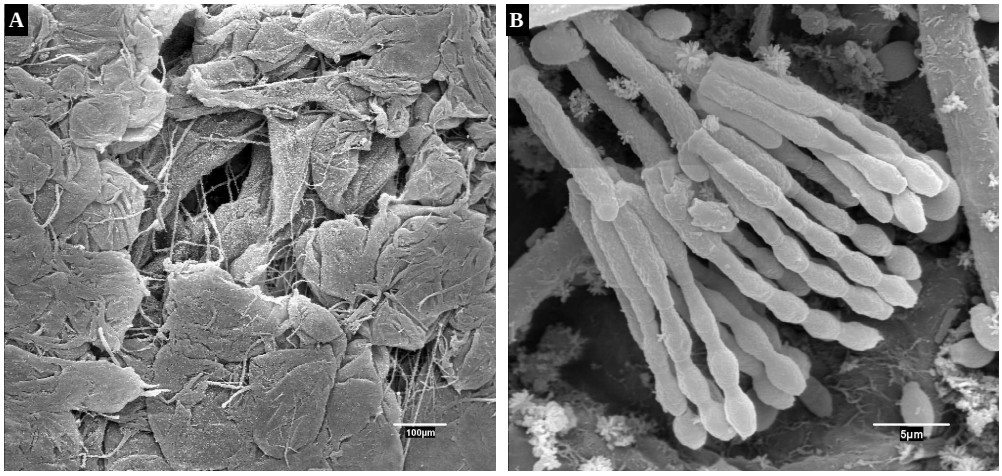

Figure 4.21. SEM micrographs of apple wounds inoculated with *P. expansum* spores and incubated for 5 days. **A:** Mycelium invading apple wound surface. **B:** High magnification of conidiophores of *P. expansum*.

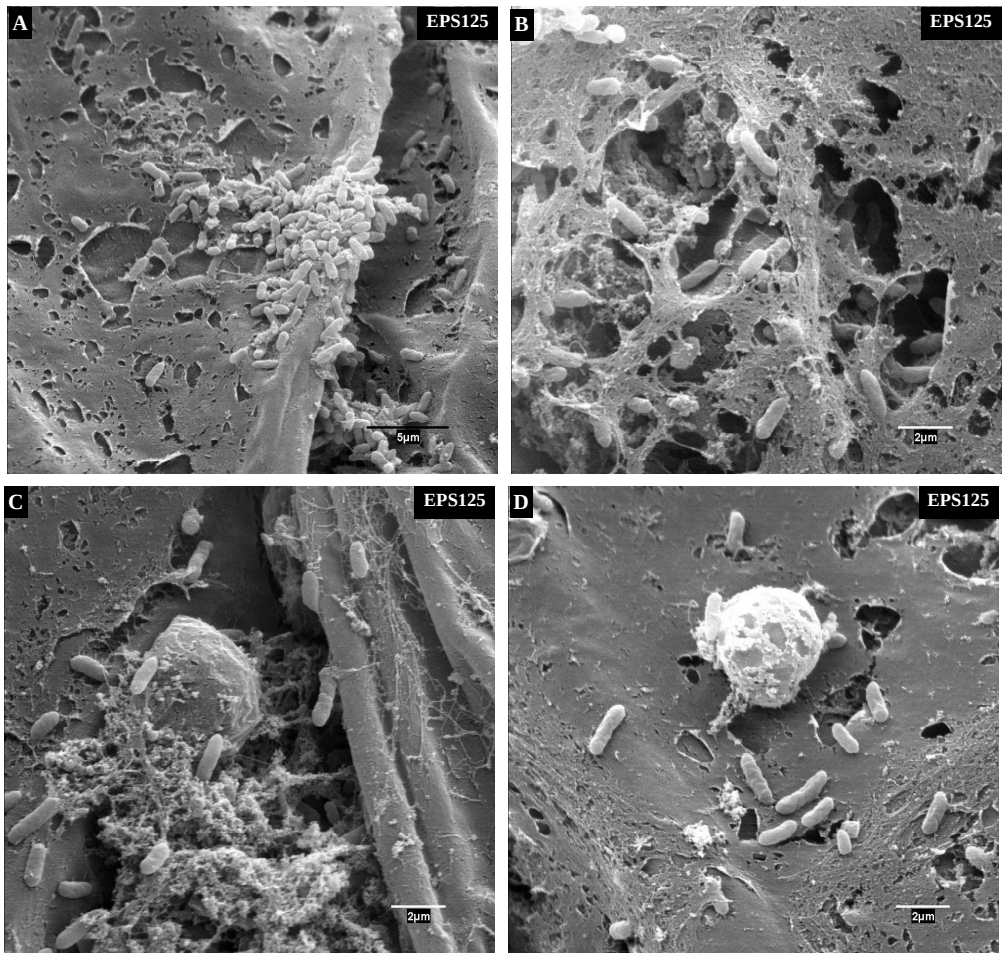

Figure 4.22. SEM micrographs of apple wounds containing *P. expansum* spores and cells of EPS125 incubated for 5 days. **A and B:** Cellular agglomerations of strain EPS125. **C and D:** EPS125 cells surrounding pathogen spores.

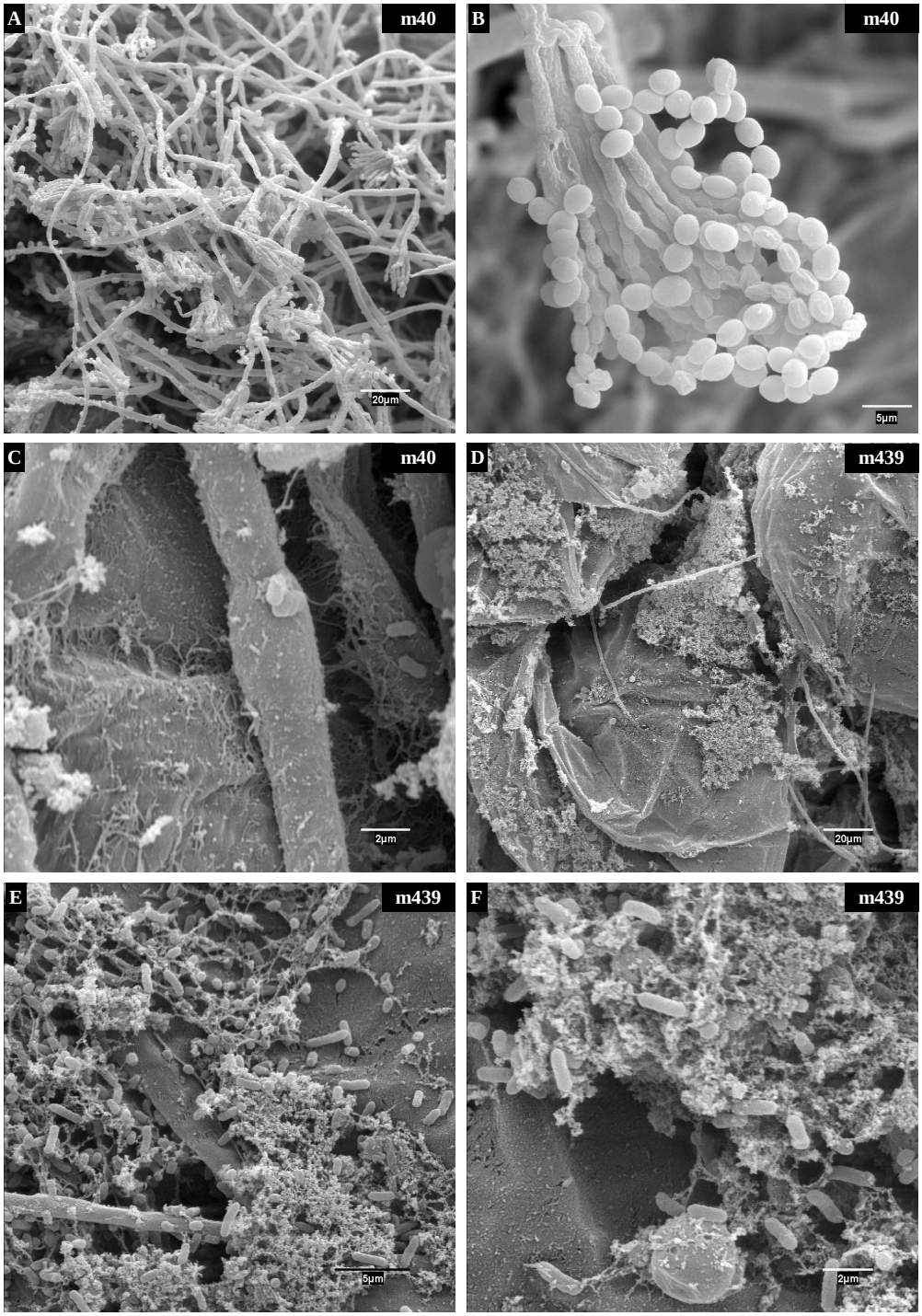

Figure 4.23. SEM micrographs of apple wounds containing *P. expansum* spores and mutant cells incubated for 5 days. **A and B:** Mycelium and conidiophores formed by *P. expansum* when was incubated together with m40 cells. **C:** m40 cells and salt precipitates placed on apple surface. **D:** m439 cells colonizing the apple surface and germ tubes of *P. expansum* invading the fruit tissue. **E:** Cellular agglomerates of the mutant m439. **F:** m439 cells interacting with conidia.

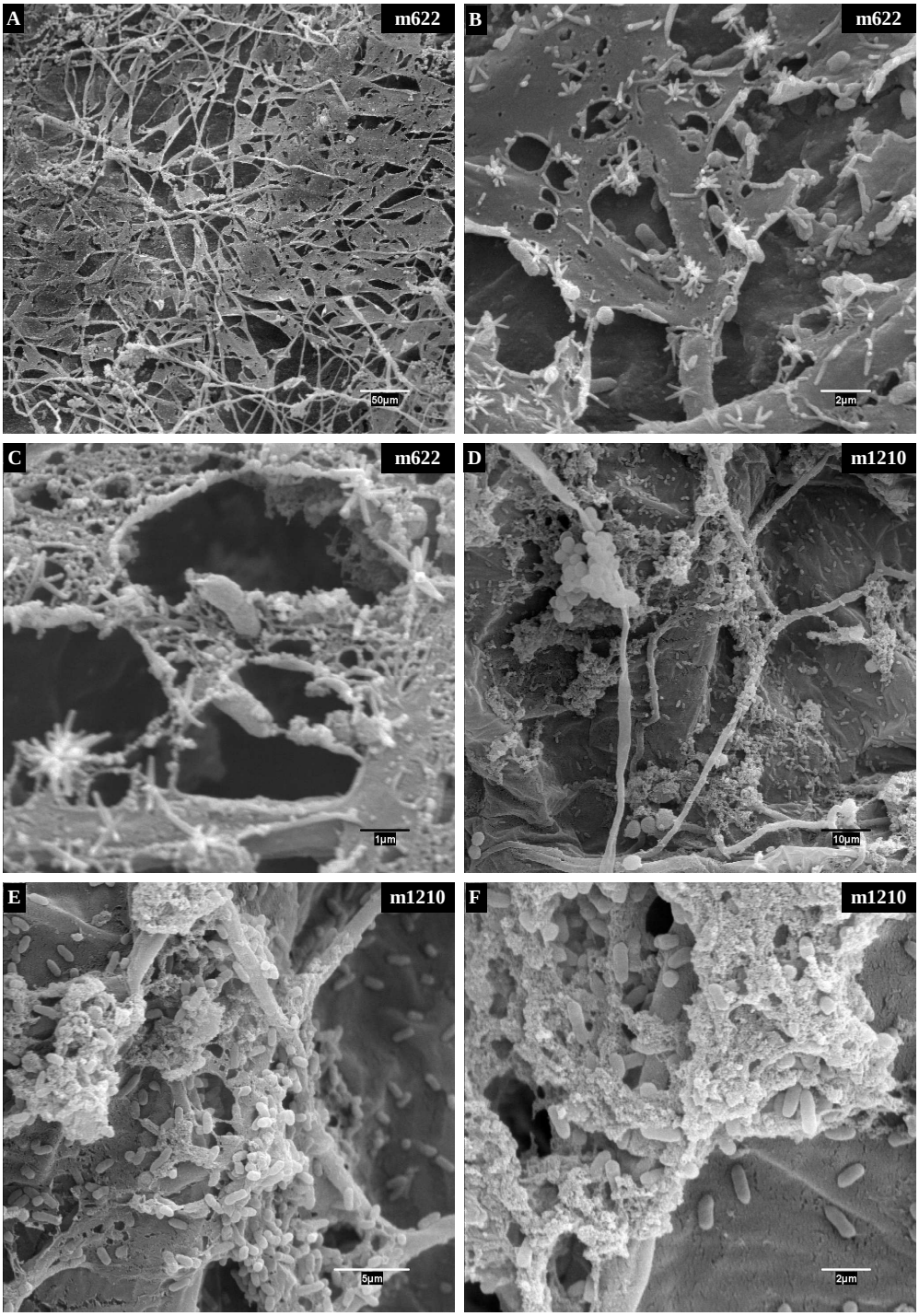

Figure 4.24. SEM micrographs of apple wounds containing *P. expansum* spores and mutant cells incubated for 5 days. **A:** Mycelium formed by *P. expansum* in contact with m622 cells. **B and C:** Cells of m622 and star-shaped salt precipitates placed on the apple wound surface. **D:** Pathogen spore in contact with cells of mutant m1210 agglomerated and linked by PLS. **E and F:** Bacterial cells of m1210 surrounding the apple surface and the germ tubes of the pathogen.

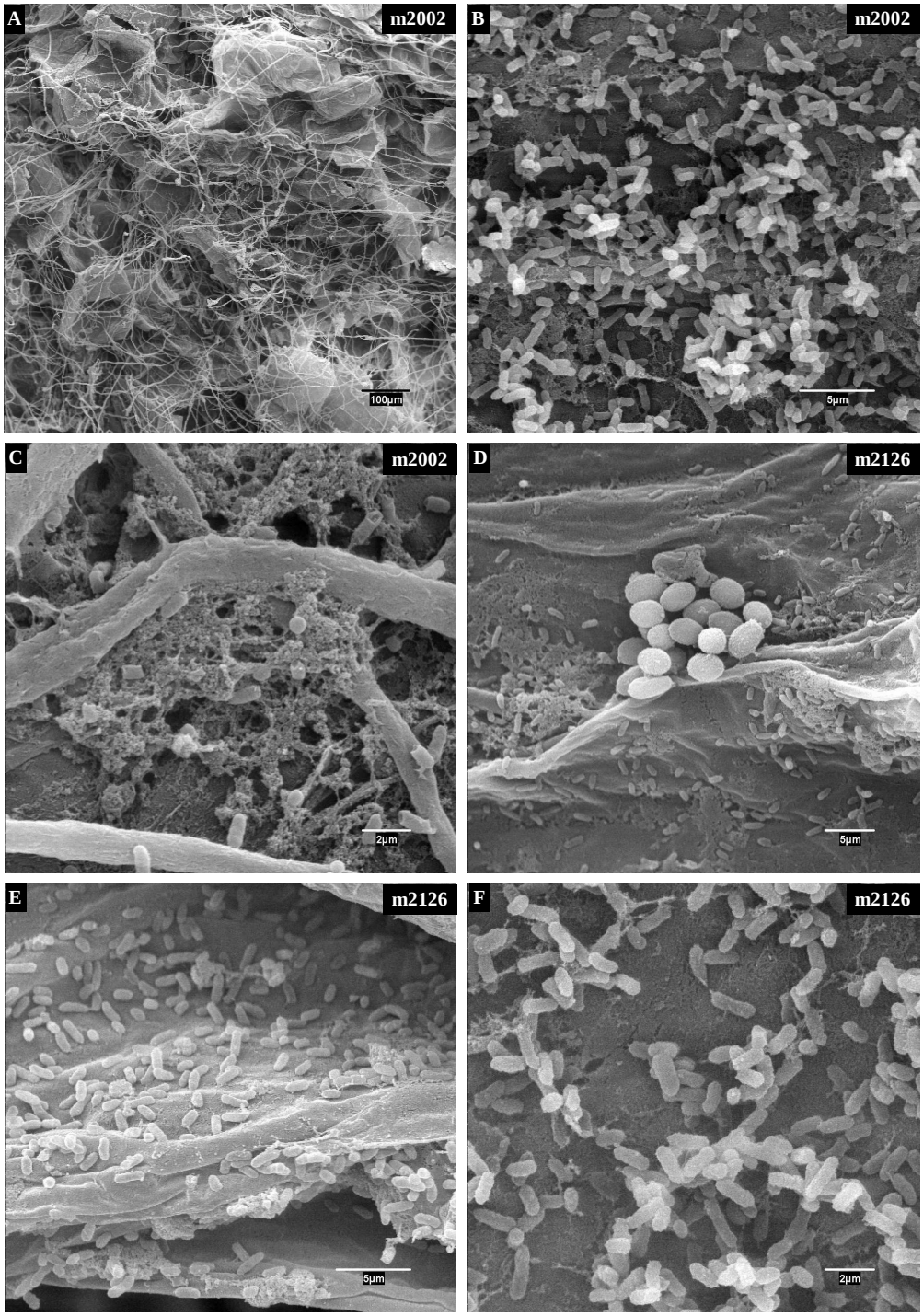

Figure 4.25. SEM micrographs of apple wounds containing *P. expansum* spores and mutant cells incubated for 5 days. **A:** Mycelium of *P. expansum* invading the apple tissue after incubation period with m2002 cells. **B:** m2002 cells adhered to the apple surface. **C:** Bacterial cells of m2002 surrounding the apple surface and the germ tubes of the pathogen. **D:** Pathogen spores in contact with m2126 cells. **E and F:** m2126 cells colonizing the apple wound surface.

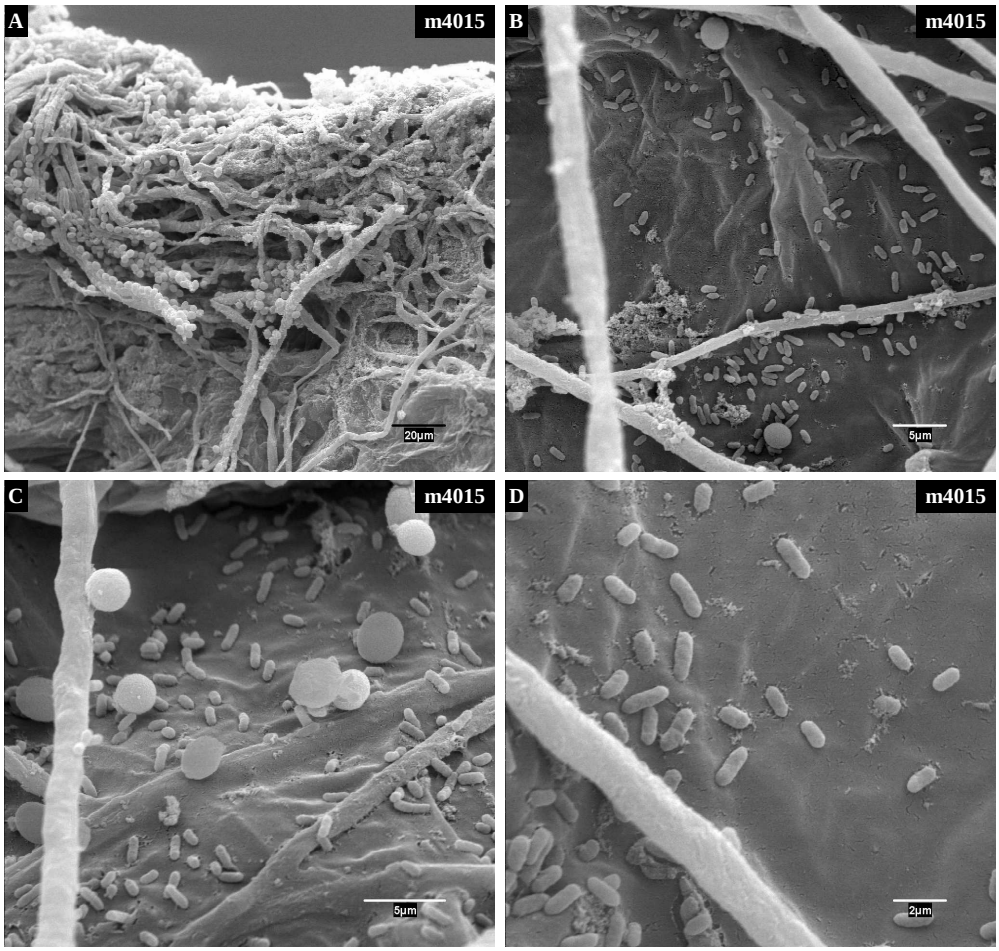

Figure 4.26. SEM micrographs of apple wounds containing *P. expansum* spores and cells of m4015 incubated for 5 days. **A:** Mycelium of *P. expansum* invading the apple tissue after incubation period with m4015 cells. **B and C:** Bacterial cells of m4015 surrounding the apple surface and germ tubes of the pathogen invading the apple tissue. **D:** Cells of m4015 adhered to apple wound surface.

Transmission Electron Microscope observations

Cultures of *P. agglomerans* EPS125 and mutants m2126 and m4015 grown in apple juice were observed by means of TEM.

Transmission electron micrographs of ultrathin sections stained with ruthenium red of strain EPS125 and mutant m2126 showed PLS linking cells and layers surrounding the cell aggregates or symplasmata (Figure 4.27). However, the presence of symplasmata groups seemed to be less frequent in case of mutant.

Negative staining micrographs of mutants m2126 and m4015 grown in apple juice showed a large amount of long PLS interconnecting cells similar to PLS formed by strain EPS125 (Figure 4.28, 4.29). In all cases, cells were grouped making difficult to take pictures.

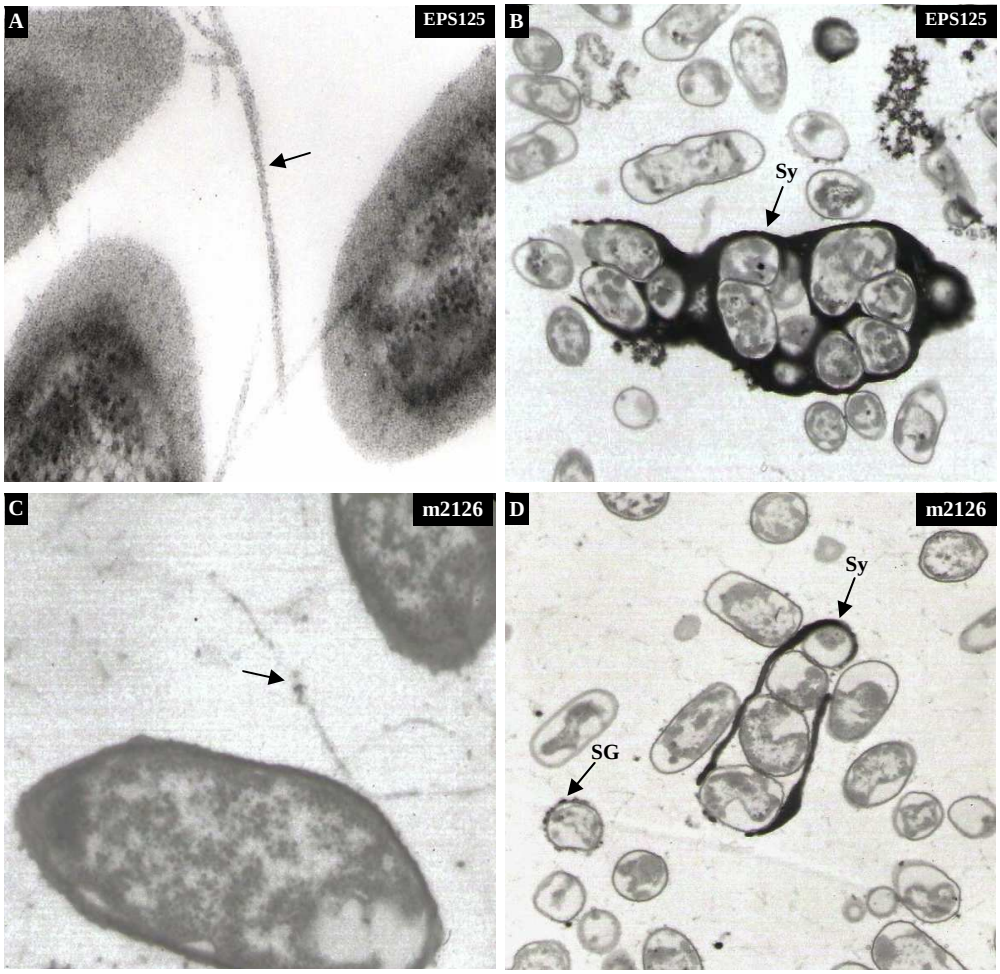

Figure 4.27. TEM micrographs of ultrathin sections stained with ruthenium red of EPS125 and m2126 cultures grown in apple juice. **A:** EPS125 cells interconnected with PLS (80,000X). **B:** Symplasmata of strain EPS125 (10,000X). **C:** m2126 cells linked by PLS (50,000X). **D:** Symplasmata of mutant m2126 (16,000X). Black arrows: PLS. SG (arrows): Saccharic granules. Sy (arrows): Symplasmata.

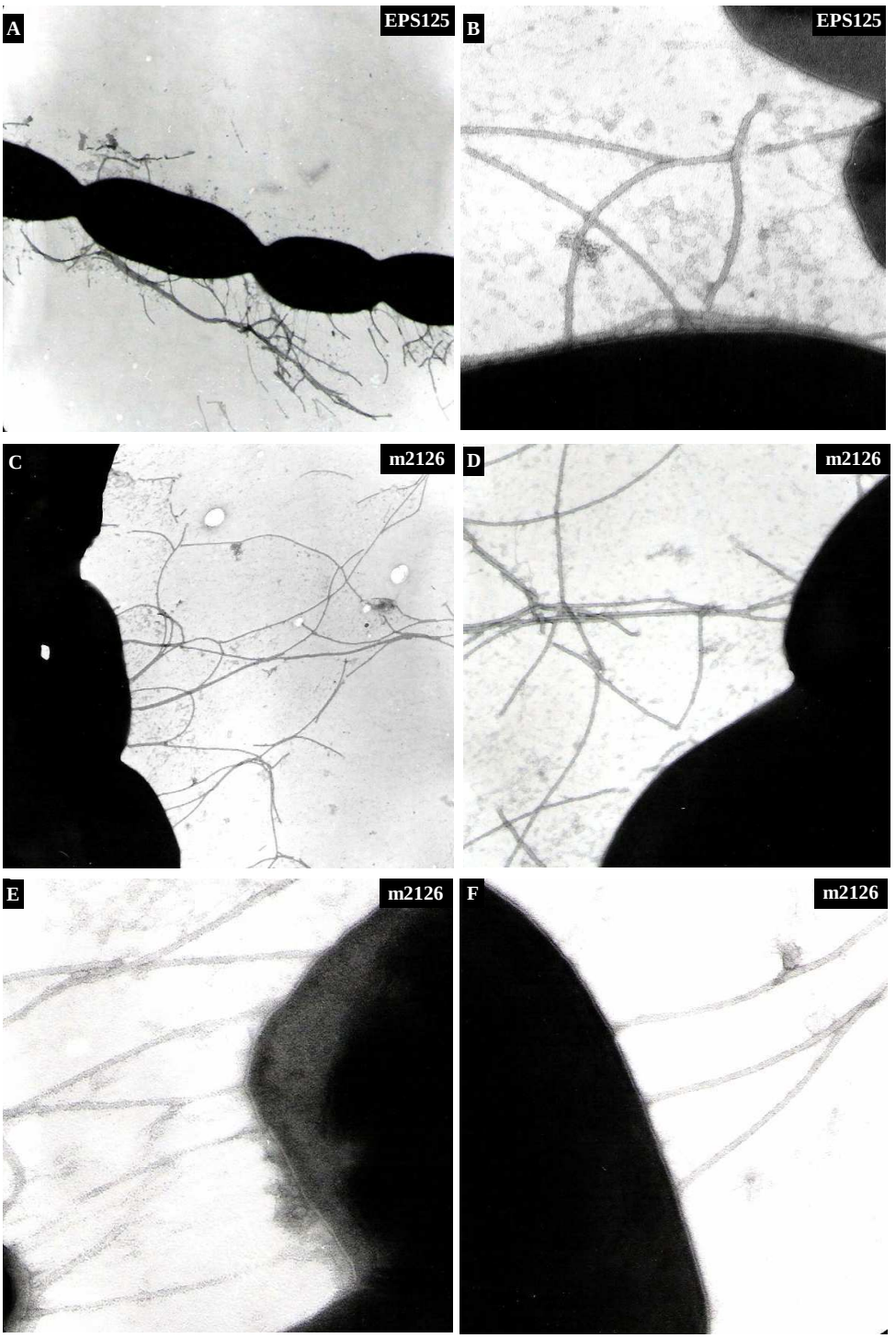

Figure 4.28. Negative staining micrographs of strain EPS125 and m2126 mutant grown in apple juice. **A:** Chain of EPS125 cells with a large amount of long PLS (16,000X). **B:** High magnification of PLS of EPS125 (100,000X). **C, D:** Long PLS of mutant m2126 (20,000X, 50,000X). **E, F:** High magnification of PLS of mutant m2126 (100,000X).

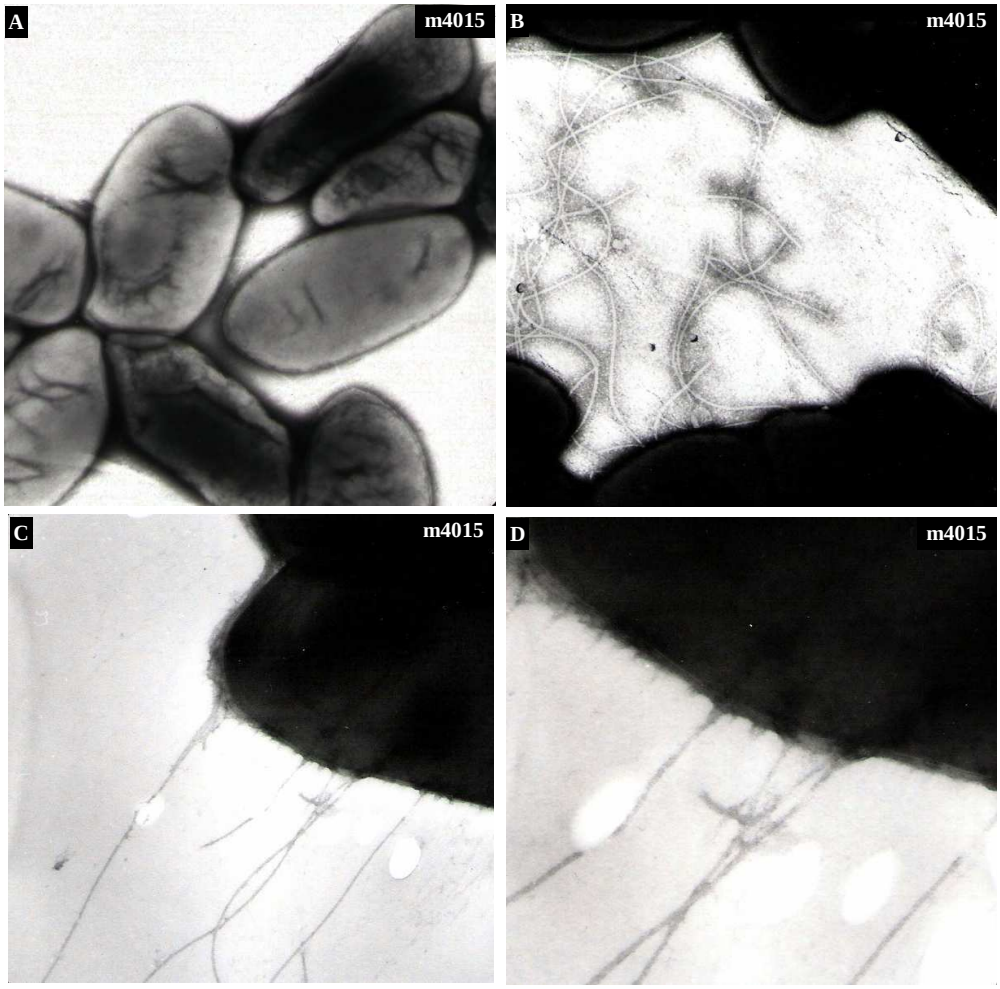

Figure 4.29. Negative staining micrographs of strain EPS125 and m4015 mutant grown in apple juice. **A and B:** Cells of mutant m4015 linked with PLS (20,000X). **C and D:** High magnification of PLS of mutant m4015 (40,000X, 100,000X).

4.2.3. Genetic analysis of mutants

Genetic analysis of disrupted sequence of selected mutants was performed with the aim to identify genes or gene products that may be related with biocontrol mechanisms used by strain EPS125.

Detection of pCAM140 by PCR

The detection of pCAM140 plasmid in selected mutants was performed by means of PCR using two specific primers designed from pCAM140 plasmid and transposon sequences. An amplification signal of 2 kb of size was obtained from genomic DNA extraction of *E. coli* S17.1-λpir (pCAM140) and from purified plasmid pCAM140. m40, m439, m622, m1210, m2002, m2126 and m4015 mutants did not show amplification signal indicating that they have minitransposon inserted into the genome (Figure 4.30).

Figure 4.30. Detection of pCAM140 plasmid. (M) λ*HindIII* marker, (1) m40, (2) m439, (3) m622, (4) m1210, (5) m2002, (6) m2126, (7) m4015, (8) EPS125, (9) *E. coli* S17.1-λpir (pCAM140), (10) pCAM140 plasmid.

Determination of the number of transposon insertions

The number of transposon insertions was determined by southern blotting and hybridization of genomic DNA digested with *AccI* and *SpeI* restriction endonucleases with two probes corresponding to Sm/Spc and GUS genes.

A single hybridized fragment of the same size for two probes was observed for the mutants m396 (4.3 kb), m439 (5.5 kb), m622 (4.5 kb), m789 (4.3 kb), m1210 (7.5 kb), m2002 (6 kb), m2126 (5), m3300 (20 kb), m3469 (4.3 kb), m3856 (7 kb) and m4015 (4.3 kb). Two hybridized fragments were detected in mutants m3152 (5 and 2 kb), m3427 (6.6 and 4 kb) and m3891 (4.4 and 2kb). The m40 mutant showed three hybridized fragments of 9, 6 and 4 kb of size making it a bad candidate to be studied. Because of m2196 mutant had multiple hybridization signal with Sm/Spc probe but any hybridization fragment with GUS probe was observed, it was discarded for further studies. The unspecific signal obtained in some mutants may be related with an excessive time of developing (Figure 4.31).

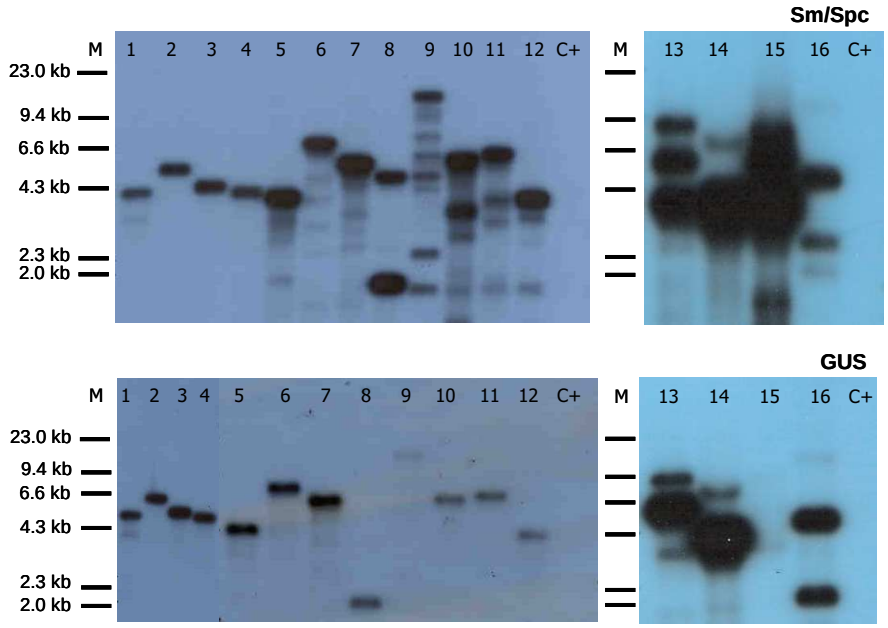

Figure 4.31. Southern blot of genomic DNA digested with *AccI* and *SpeI* enzymes and hybridized with Sm/Spc (upper) and GUS (lower) probes. (M) λ*HindIII* marker, (1) m396, (2) m439, (3) m622, (4) m3469, (5) m789, (6) m1210, (7) m2002, (8) m3152, (9) m3300, (10) m3427, (11) m3856, (12) m4015, (13) m40, (14) m2126, (15) m2196, (16) m3891, (C+) EPS125.

Sequencing of transposon flanking regions

Interesting hybridized fragments in southern blot analysis were subcloned in pBluescript SK (+) (pBS) plasmid and transformed into *E. coli* XL1-Blue. Only recombinant clones containing pBS plasmid bound to hybridized fragments from mutants m40, m2002, m2126 and m4015 were obtained after incubation in LB plates supplemented with Amp (100 μg/ml), Sm (10 μg/ml) and Spc (25 μg/ml). The plasmids extracted were named pBS-40, pBS-2002, pBS-2126 and pBS-4015, respectively. *E. coli* XL1-Blue cells transformed with DNA controls (pBS and pBS-125) grew in LB plates supplemented with Amp (100 μg/ml) and did not grow in LB plates supplemented with Amp (100 μg/ml), Sm (10 μg/ml) and Spc (25 μg/ml), indicating that they harbour the corresponding plasmids (pBS and pBS-125) but not the minitransposon GUS. The negative control of *E. coli* XL1-Blue cells transformed with water did not grow in any plate as it was expected.

The presence of pBS-40, pBS-2002, pBS-2126 and pBS-4015 plasmids carrying hybridized fragments was confirmed by digestion with *KpnI* and *SacI* enzymes. The pBS vector of 3 kb and inserts of 6, 5.5, 5.5 and 7 kb of size were released from pBS-40, pBS-2002, pBS-2126 and pBS-4015 respectively, maintaining approximately the size showed in southern blot analysis.

The fragments comprised between pBS vector and transposon regions of pBS-40, pBS-2002, pBS-2126 and pBS-4015 were sequenced. DNA and putative translated protein sequences were analyzed and related nucleotide and aminoacid sequences were identified in the GenBank database (Table 4.4).

Table 4.4. Analysis of the flanking sequences to transposon in *P. agglomerans* EPS125 mutants defectives in biocontrol.

Mutant	AC	Id	*E* value	Gene/Protein
m40	CAH04858	35 %	1e-10	Dihydrodipicolinate Synthase protein (DHDPS) of archaeon
	AAL42037	29 %	2e-09	DHDPS of *Agrobacterium tumefaciens* C58
	CAF30132	32 %	2e-09	DHDPS of *Methanococcus maripaludis* S2
		30 %	2e-08	DHDPS/N-acetylneuraminate lyase of archaeon GZfos26D8
	BAC97608	26 %	3e-06	Putative DHDPS of *Vibrio vulnificus* YJ016
m2002				No match
m2126	BAC22645	33 %	1e-17	Hypothetical protein of *Comamonas* sp. NCIMB 9872
m4015	ZP_00568773	41 %	3e-82	L-lysine 6-monooxygenase (NADPH) of *Frankia* sp. EAN1pec
	CAB53328	40 %	9e-81	Putative peptide monooxygenase *Streptomyces coelicolor* A3(2)
	AAZ55902	39 %	1e-74	Putative peptide monooxygenase of *Thermobifida fusca* YX
	ZP_00861680	38 %	6e-72	L-lysine 6-monooxygenase (NADPH) of *Bradyrhizobium* sp. BTAi1
	ZP_00501498	37 %	8e-69	Lysine/ornithine N-monooxygenase of *Burkholderia pseudomallei* S13
	ZP_00417730	36 %	2e-69	L-ornithine N5-oxygenase of *Azotobacter vinelandii* AvOP
	AAG27518	38 %	2e-68	L-ornithine N5-hydroxylase of *Pseudomonas* sp. B10

AC: Accesion Code to GenBank database. **Id:** Identity percentage (nucleotide and aminoacid sequences). ***E*value:** Parameter that describes the number of hits one can "expect" to see just by chance when searching a database of a particular size.

The sequence of 1814 b obtained from pBS-40 (annex II) did not show similarity with any DNA sequence deposited in the GenBank database. However, the protein sequence deduced had similarity with aminoacid sequence of Dihydrodipicolinate Synthase (DHDPS) proteins. The DNA sequence from 1565 to 1254 b showed similarity with DHDPS proteins of archaeon, *A. tumefaciens* C58 and *M. maripaludis* S2. The submitted sequence from 1222 to 701 b also showed identity with a putative DHDPS of *V. vulnificus* YJ016. Any other similar sequence was found for the remaining DNA sequence in the GenBank database (Figure 4.32).

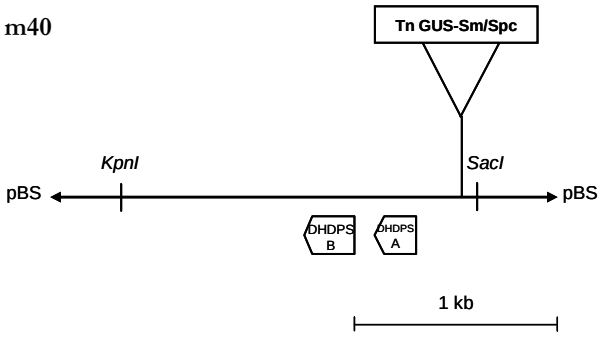

Figure 4.32. Physical map of insert of pBS-40 placed between *KpnI* and *SacI* target sequences of pBS plasmid. The insertion point of minitransposon carrying GUS and Sm/Spc genes is marked. The orientation of the arrows indicates the direction of gene transcription. The deduced aminoacid sequence from insert sequence of mutant m40 showed identity with DHDPS proteins of archaeon, *A. tumefaciens* and *M. maripaludis* (DHDPS A) and of *V. vulnificus* (DHDPS B).

The flanking sequence to transposon obtained from pBS-2002 of 1652 b (annex II) did not show any similarity with any nucleotide and aminoacidic sequences deposited in GenBank database. However, this sequence (from 77 to 938 b) showed a large similarity with IS1222 insertion sequence of *E. coli* E223 (90 % identity); with DNA sequence of *tnpA* gene of *Salmonella enterica* (90 % identity) and its gene product, a hypothetical protein B (88 % identity); with IS1222 sequence of the indigenous plasmid pEA9 of *E. agglomerans* 339 (89 % identity); with IS1222 insertion sequence (*orf*A and *orf*B) of *Rahnella aquatilis* (88 % identity) and its *orf*B gene product, a orfB protein (88 % identity); with orfB protein of *P. agglomerans* pv. *gypsophilae* (85 % identity); with an IS1222-like transposase of *Yersinia enterocolitica* (85 % identity); and with a possible transposase remnant YPCD1.04 of *Yersinia pestis* CO92 (84 % identity) (Figure. 4.33).

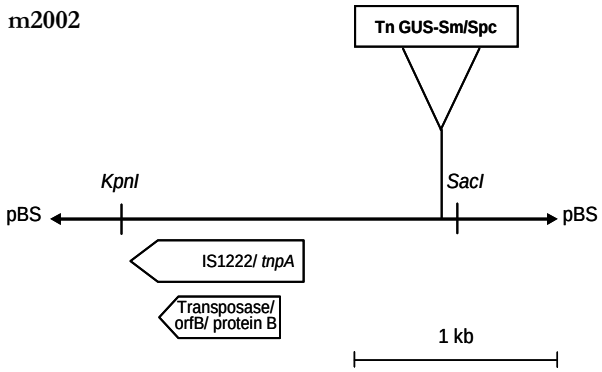

Figure 4.33. Physical map of insert of pBS-2002 placed between *KpnI* and *SacI* target sequences of pBS plasmid. The insertion point of minitransposon carrying GUS and Sm/Spc genes is marked. The orientation of the arrows indicates the direction of gene transcription. DNA sequence of mutant m2002 showed identity with IS1222 insert sequences of *E. coli*, *E. agglomerans*, *R. aquatilis* and with *tnpA* gene of *S. enterica*. The deduced aminoacidic sequence showed identity with orfB protein of *R. aquatilis* and *P. agglomerans*, with hypothetical protein B of *S. enterica*, and with a transposase of *Y. pestis* and *Y. enterocolitica*.

The sequence of 1473 b obtained from insert of pBS-2126 (annex II) did not show identity with any DNA sequence deposited in the GenBank database. Nevertheless, the flanking to transposon DNA sequence from 876 to 1382 b had a large similarity with a hypothetical protein of *Comamonas* sp. NCIMB 9872 of unknown function. In addition, the protein sequence deduced from DNA sequence from 48 to 753 b showed similarity with bacterial luciferase family proteins of *P. syringae* pv. tomato DC3000 (55 % identity), *P. syringae* pv. syringae B728a (52 % identity) and *P. syringae* pv. phaseolicola 1448A (53 % identity); with a putative monooxygenase protein of *B. bronchiseptica* RB50 (47 % identity); and with a hypothetical protein ISM_05230 of *R. nubinhibens* ISM related with a coenzyme F420-dependent N5,N10-methylene tetrahydromethanopterin reductase and related flavin-dependent oxidoreductases (42 % identity) (Figure 4.34).

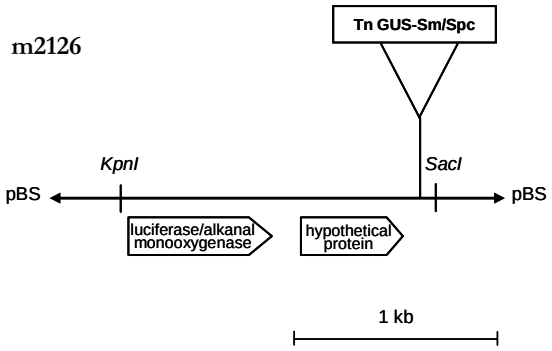

Figure 4.34. Physical map of insert of pBS-2126 placed between *KpnI* and *SacI* target sequences of pBS plasmid. The insertion point of minitransposon carrying GUS and Sm/Spc genes is marked. The orientation of the arrows indicates the direction of gene transcription. The deduced aminoacid sequence of mutant m2126 showed identity with bacterial luciferase family proteins of *P. syringae*; with an alkanal monooxygenase protein of *B. bronchiseptica* and with a hypothetical protein ISM_05230 of *R. nubinhibens*. The DNA sequence also showed identity with a hypothetical protein of *Comamonas* sp. (hypothetical protein).

The sequence obtained from pBS-4015 of 3619 b (annex II) had 861 b (from 135 to 996 b) exactly the same as insert of pBS-2002 noted above. This showed the same identity and identity percentages with IS1222 insertion sequence of *E. coli* E223; with DNA sequence and gene product of *tnpA* gene of *S. enterica*; with IS1222 sequence of the indigenous plasmid pEA9 of *E. agglomerans* 339; with IS1222 insertion sequence (orfA and orfB) of *R. aquatilis* and its orfB gene product; with a possible transposase remnant YPCD1.04 of *Y. pestis* CO92; with orfB protein of *P. agglomerans* pv. *gypsophilae*; and with IS1222-like transposase of *Y. enterocolitica*. The translated flanking to transposon sequence from 2002 to 3279 b showed identity with an L-lysine 6-monooxygenase (NADPH) of *Frankia* sp. and *Bradyrhizobium* sp. BTAi1; with a putative peptide monooxygenase of *Streptomyces coelicolor* A3(2) and *Thermobifida fusca* YX; with a Lysine/ornithine N-monooxygenase of *Burkholderia pseudomallei* S13; with a L-ornithine N5-oxygenase of *Azotobacter vinelandii* AvOP; and with a L-ornithine N5-hydroxylase of *Pseudomonas* sp. B10 (Figure 4.35).

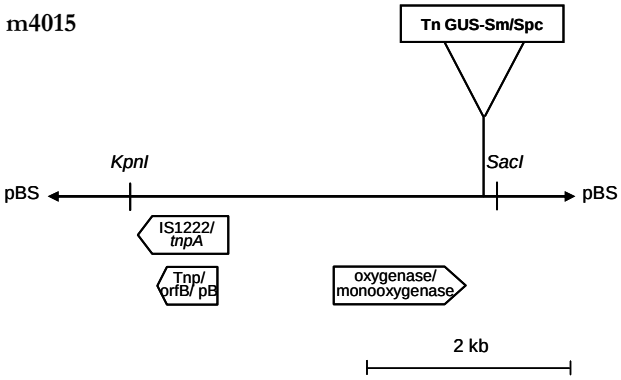

Figure 4.35. Physical map of insert of pBS-4015 placed between *KpnI* and *SacI* target sequences of pBS plasmid. The insertion point of minitransposon carrying GUS and Sm/Spc genes is marked. The orientation of the arrows indicates the direction of gene transcription. DNA sequence of mutant m4015 showed identity with IS1222 insert sequences of *E. coli*, *E. agglomerans*, *R. aquatilis* and with *tnpA* gene of *S. enterica*. The deduced aminoacid sequence showed identity with an orfB protein of *R. aquatilis* and *P. agglomerans*, a hypothetical protein B (pB) of *S. enterica*, and a transposase (Tnp) of *Y. pestis* and *Y. enterocolitica*. The translated sequence also had similarity with oxygenase and monooxygenase and related proteins of *Frankia* sp., *Bradyrhizobium* sp., *S. coelicolor*, *T. fusca*, *B. pseudomallei*, *A.r vinelandii* and *Pseudomonas* sp.

Transformation of *P. agglomerans* EPS125 by electroporation

A transformation protocol for *P. agglomerans* EPS125 by electroporation was fine-tuned having into account the quantity of DNA to transform and the growth, wash and electroporation conditions.

The most important factor to obtain recombinant colonies of strain EPS125 was the growth condition. A greater number of EPS125 cells acquired the pBBR1MCS-3 plasmid that conferred resistance to tetracycline when host cells were grown in LB agar than were incubated in LB broth. In addition, a much more effectively of electroporation was observed when cells were washed with 1 mM HEPES pH6.8 with 10 % glycerol solution and electrocompetent cells were transformed with 1 μg of DNA at 2.5 kv/cm, 200 Ω and 25 μF.

5. Discussion

Knowledge of biocontrol mechanisms used by a biological control agent (BCA) allows establishing the optimum conditions for the interaction with the pathogen (Spadaro and Gullino, 2004). However, the study and understanding of these mechanisms of action are a difficult task due to the complex interactions occurring between BCA, pathogen, host, environmental conditions and other microorganisms present.

Several biocontrol mechanisms depending on the BCA have been described in biocontrol of postharvest diseases including antibiosis (Gueldner *et al.*, 1988; Janisiewicz *et al.*, 1991; Edwards and Seddon, 2001), induced resistance in the host tissue (El Ghaouth *et al.*, 2001; Janisiewicz *et al.*, 2003), direct interaction (Castoria *et al.*, 2001), quorum quenching, and competition for nutrients (Wisniewski *et al.*, 1989; Mari *et al.*, 1996; Filonow, 1998; Bonaterra *et al.*, 2003), and space (Andrews *et al.*, 1994; Pasichnyk *et al.*, 2005).

In the present work, to determine the biocontrol mechanism used by *Pantoea agglomerans* EPS125 against *Penicillium expansum* in a first step a phenotypic characterization including antibiosis, direct interaction, competition for nutrients and space has been performed by means of *in vitro* and *ex vivo* assays.

So far, most works related with phenotypic characterization of biocontrol mechanism are based on the detection of antimicrobial compounds by means of *in vitro* assays, which are easy and rapid to perform (Wright *et al.*, 2001). However, cases where *in vitro* did not correlate with *in vivo* assays have been found (Andrews, 1992; Bonaterra, 1997; Ellis *et al.*, 2000; Ji and Wilson, 2002). For example, it has been observed that *Lysobacter enzymogenes* C3, BCA of Bipolaris leaf spot of tall fescue and Pythium damping-off of sugar beet, produces extracellular β-1,3-glucanases capable of degrading the cell walls of fungi. The triple-mutant of *L. enzymogenes* C3, strain G123, which has the three glucanase genes mutated, was significantly reduced in biological control activity against Bipolaris leaf spot of tall fescue and Pythium damping-off of sugar beet. However, it was not affected in antimicrobial activity toward *Bipolaris sorokiniana* and *Pythium ultimum* var. *ultimum* using *in vitro* assays. Other studies performed on strain C3 also indicate the influence of multiple mechanisms on biological control in this system that can not be detected by means of *in vitro* assays (Palumbo *et al.*, 2005). In addition, the composition of the culture media used in *in vitro* assays has showed to be decisive for the synthesis of antimicrobial compounds obtaining variable results depending on the media chosen. For example, the antagonistic activity of *Erwinia herbicola* towards *Eutypa lata* on grape wood agar was enlarged maximally by adding 0.5% glucose or 1-2% peptone to culture media, and it was reduced by the addition of nitrate and phosphate salts on glucose-supplemented medium (Schmidt *et al.*, 2001). For these facts, it is important to complement the results of *in vitro* assays with other approaches nearly approximate to *in vivo* conditions that deal with the production of antimicrobial compounds.

In the case of *P. agglomerans* EPS125, the absence of detection of antifungal compounds production against *P. expansum* has been reported already using dual culture assays with different growth media (Vázquez, 1998). This result coincides with findings obtained in the present work with a system more similar to *in vivo* conditions. Conidia of *P. expansum* were able to germinate when were incubated with spent cell-free culture filtrates of strains EPS125, EPS484 and SBW25 grown in apple juice, indicating that strain EPS125 does not produce, or not in enough amount antifungal substances that inhibit the pathogen spore germination.

The production of chitinases by some BCA has a visible effect on the fungal growth as has been reported in several works (Chernin *et al.*, 1995; Zhang and Yuen, 2000). Nonetheless, *P. agglomerans* EPS125 has showed neither taxis to spores and germ tubes of *P. expansum*, nor chitinolytic activity. Therefore, in the conditions tested it is unlikely that parasitic events

mediated by attachment and production of chitinases be important in the biocontrol of blue mould.

In comparison with antibiosis, there are few studies about competition for nutrients and even less on competition for space as the main biocontrol mechanism because of the limitation in methods available to evaluate its importance on disease suppression. However, several reports on the interaction between epiphytic microorganisms showed that bacteria and yeast are able to take up nutrients from diluted solutions more rapidly and in a greater quantity than the germ tubes of fungal pathogens can (Brodie and Blakeman, 1976). This may result in a marked reduction of nutrients available to the pathogen restricting spore germination and its hypha development. For instance, it has been suggested that competition for nutrients may be important in the control of *Botrytis cinerea* and *P. expansum* by *P. agglomerans* B66 and B90 on apple (Bryk *et al.*, 1998), and in the control of *P. expansum* by *E. herbicola,* which exhausts the aminoacids contained in the medium (Janisiewicz *et al.*, 2000).

The method described by Janisiewicz *et al.* (2000) to study competition for nutrients without the interference of competition for space has been used with *P. agglomerans* EPS125 and *P. expansum*. Results showed that competition for nutrients, as well as the production of antifungal compounds are not the mechanisms responsible of EPS125 biocontrol activity as pathogen spores germinated when were physically separated from cells of EPS125 by a filter. On the contrary, the inhibition of germination of pathogen conidia was only achieved when there was cell-to-cell interaction. This agrees with results obtained by Bonaterra *et al.* (2003) in the biocontrol of *Monilinia laxa* and *Rhizopus stolonifer* by *P. agglomerans* EPS125 in stone fruit. The authors showed that preemptive exclusion of pathogens by wound colonization and cell-to-cell interaction with pathogen spores appeared to be the main biocontrol mechanisms of strain EPS125.

The characterization of the extracellular material produced by EPS125 that may be related with its biocontrol ability was accomplished by means of Infrared (IR) spectroscopy. IR spectroscopy is a non-destructive technique that allows the rapid and qualitative characterization of structural features and identification of polymeric material such as different types of exopolysaccharides (Bellamy, 1958; Parker, 1971; Griffiths, 1975). The three IR spectra of strain EPS125 grown in LB broth, osmoadapted medium (SDM) and apple juice shared four bands at 1650, 1550, 1405 and 1090 cm^{-1} corresponding to amide I, amide II, carboxylate ion and P-O and C-OH stretching, respectively. These bands are related with the presence of peptides and proteins in samples, rather, with a cellular growth of strain EPS125. However, unlike LB spectrum, SDM and apple juice spectra showed a 1060 cm^{-1} band associated with the presence of alginate polysaccharide.

The bacterial product alginate, which is involved in biofilm formation by *Pseudomonas aeruginosa* in pulmonary infections and in industrial water systems (Stoodley *et al.*, 2002), is a exopolysaccharide (EPS) composed of β-D-mannuronic acid and the C-5 epimer α-L-guluronic acid linear (1→4)-linked (Evans and Linker, 1973). In general, it has been proposed that EPS play an important role in initial adhesion and anchoring of bacteria to solid surfaces (Ramphal and Pier, 1985; Marcus and Baker, 1985; Mai *et al.*, 1993; Stoodley *et al.*, 2002). EPS may help overcome the electrostatic repulsion that exists between the cell and the surface which usually is negatively charged, leading to irreversible adherence of cell to the substratum (Characklis, 1990; Marshall, 1992). In addition, EPS can protect bacteria from dehydration as it can incorporate large amounts of water into its structure by hydrogen bonding (Donlan, 2002). In a study performed by Ophir and Gutnick (1994), the role of EPS in protection from desiccation was examined. In these experiments, mucoid strains of *Escherichia coli*, *Acinetobacter calcoaceticus*, and *Erwinia stewartii* were compared to nonmucoid variants in their resistance to desiccation. It was observed that mucoid strains of all three bacteria showed better survival under conditions of dehydration (Davey and O'toole, 2000). The EPS matrix also has the potential to physically

prevent access of certain antimicrobial agents into the biofilm such as metals, cations, antibiotics and toxins by binding to compounds and/or by inactivating them (Decho and Moriarty, 1990; Bayer *et al.*, 1991; Bayer *et al.*, 1992; Flemming, 1993; Donlan, 2000). Besides the ability to restrict diffusion of compounds from the surrounding milieu into the biofilm (Gilbert *et al.*, 1997), it has been reported that EPS supply protection from a variety of environmental stresses, such as UV radiation, pH shifts and osmotic shock (Flemming, 1993), as well as provide the close proximity of the bacteria to one another allowing the genetic exchange and enhancing the plasmid stability (Van Elsas *et al.*, 1988; Boyd and Chakrabarty, 1995).

EPS production by bacteria is known to be affected by nutrient composition of the growth medium. The excess of available carbon and the limitation of nitrogen, potassium, or phosphate found in apple juice (Waites *et al.*, 2001) promote EPS synthesis (Sutherland, 2001). Apart from nitrogen limitation, alginate production by *P. aeruginosa* is also up-regulated during membrane perturbation induced by ethanol, when cells are exposed to media of high osmolarity (Devault *et al.*, 1990), or under conditions of high oxygen tension (Anwar *et al.*, 1989; Davies and Geesey, 1995). Therefore, according to other reports, it seems that strain EPS125 produces alginate polysaccharide under environmental stresses, such as nitrogen limitation in apple juice and high osmolarity in SDM medium, and do not when it grows in LB rich medium. In addition, it has been reported that osmotic stress yields significant physiological changes consisting of intracellular accumulation of compatible solutes or membrane stabilisation (Csonka, 1989; Csonka and Hanson, 1991; Miller and Wood, 1996; Bonaterra *et al.*, 2005). These are synthesized by cells or taken from the growth medium to balance the osmotic pressure of the environment and maintain cell turgor (Potts, 1994; Sleator and Hill, 2002). Important representatives of this class of solutes in bacteria include K+ ions, the amino acids glutamate, glutamine, proline and alanine, the quaternary amines glycine–betaine (GB), the tetrahydropyrimidine ectoine and sugars such as sucrose and trehalose (Csonka, 1989). Bonaterra *et al.* (2005) have developed an osmoadaptation system for *P. agglomerans* EPS125 by combining saline osmotic stress (NaCl) and osmolyte (GB) amendment to the growth medium. Osmoadapted cells showed a higher tolerance to desiccation accumulating intracellular GB and were more effective in blue mould rot control than non-osmoadapted cells. Moreover, the addition of GB to the NaCl amended growth medium increased 4-5 fold growth rate improving its process of mass production. Therefore, the production of exopolysaccharides induced by growth conditions may play a role in the mechanism of biocontrol of *P. agglomerans* EPS125.

In the present work, transmission electron microscopy (TEM) has been used to examine ultrathin sections of EPS125 cells grown in LB broth and apple juice. By using the specific polysaccharide-stain, ruthenium red, a saccharic layer surrounding and enclosing EPS125 cells grown in apple juice forming cellular aggregates or symplasmata was observed. Nevertheless, this polysaccharide material was not visualized in EPS125 cultures grown in LB broth. Scanning electron microscopy (SEM) micrographs of EPS125 cultures grown in apple juice also showed symplasmatum structures, but unlike of TEM micrographs of ruthenium red-stained ultrathin sections, the polysaccharide sheath was not observed. This absence may be due to dehydrating agents used during SEM processing of samples, which are organic solvents that can extract and remove cell components of samples (Montesinos *et al.*, 1983); as well as this sheath may be intimately disposed on bacterial surfaces being undistinguishable in SEM micrographs.

Beijerinck (1988) described the characteristics of symplasmata-forming *P. agglomerans* in 1888. Symplasmata are defined as multicellular aggregate structure in which several (at least two) to hundreds of individual cells tightly bind together (Feng *et al.*, 2003). The formation of symplasmata in pure culture by *P. agglomerans* and related strains, which seems to be strain specific (Feng *et al.*, 2003), has been well documented since Beijerinck (Gilardi and Bottone, 1971; Beijerinck, 1988). The wetland rice rhizosphere bacterium *P. agglomerans* NO30 could form symplasmata only in LB liquid medium and on rice root surface; however these structures were

neither found on LB agar medium nor on wheat root surface (Achouak *et al.*, 1994). In case of the endophytic diazotrophic bacterium isolated from rice *P. agglomerans* YS19 was able to form symplasmata in liquid LB and GY (glucose yeast extract) medium, on agar medium and *in planta* (Feng *et al.*, 2003). Observations performed in this work show that *P. agglomerans* EPS125 can form symplasmata in apple juice but not in LB broth. Hence, according to other reports, these data bear out the theory suggested by Feng *et al.* (2003) that symplasmata formation of *P. agglomerans* might be strain specific.

In addition, data from IR spectroscopy and TEM observations of ultrathin sections show that alginate polysaccharide and the saccharic layer of symplasmata only were detected when strain EPS125 is incubated in apple juice but not in LB broth. Hence, this fact supports that the polysaccharide sheath that traps EPS125 cells in symplasmata might be composed of alginate polysaccharide.

According to growth conditions and nutrient source used a differential phenotypic behaviour of *P. agglomerans* EPS125 was observed. This metabolic versatility and phenotypic plasticity is related with the ability of its genotype to respond phenotypically to environmental stimuli (Costerton, 1995). Therefore, it has to take into account what experimental system of study and, even more, what nutrient source are selected to study biocontrol mechanisms used by strain EPS125 as these mechanisms may be stated only in certain growth conditions. Specifically, according to the results obtained, it was decided that the best system to analyse biocontrol mechanisms of *P. agglomerans* EPS125 is based on the use of apple juice as nutrient source in *in vitro* assays or directly the apple fruit.

Other characteristic structures observed in TEM micrographs of ultrathin sections of EPS125 cells grown in apple juice were pili-like structures (PLS) interconnecting EPS125 cells. These structures were detected more clearly and easily in negative staining TEM micrographs and SEM micrographs of overnight cultures of strain EPS125. However, considerable differences of cell and PLS sizes existed among TEM and SEM techniques. Unlike TEM technique, which maintains original sizes, it has proved that drying steps during specimen preparation by SEM protocol can cause shrinkage in bacterial cells involving a significant variation of cellular structures size (Montesinos *et al.*, 1983). Moreover, SEM processing of apple wounds containing pathogen spores and EPS125 cells presents other disadvantages. The continuous change from dehydration and fixation solutions during SEM processing of samples produces the removal of inoculated cells from apple surfaces in many observations. Also, because of the difficult accessibility inside of apple wounds by the fixative and buffer solutions, salt precipitates accumulate on specimen surfaces, thus complicating its study. Nevertheless, these disadvantages do not impinge on processing and observation of cell cultures grown in inserts of Millicell® tissue culture plates with apple juice.

The large amount of PLS observed interconnecting EPS125 cells in SEM and TEM micrographs were very similar to cell surface organelles named type IV pili, which have been found on many Gram-negative bacteria. These structures share the same physical characteristics, they are long (> 1–5 μm), thin (60–70 Å) and mechanically strong polymeric fibres (Parge *et al.*, 1995). It is well documented that type IV pili mediate a variety of functions including attachment (Cairns-Smith, 1983; Characklis, 1990; Brozel *et al.*, 1995), aggregation (Swanson *et al.*, 1971), twitching motility (Merz *et al.*, 2000; McBride, 2001), variation of target tissue specificity (Jonsson *et al.*, 1994) and competence for DNA uptake (Froholm *et al.*, 1973; Zhang *et al.*, 1992).

Although flagella are the most studied means of locomotion in bacteria (swimming motility) (Abeygunawardana *et al.*, 1991a; Abeygunawardana *et al.*, 1991b), other bacterial motility mechanisms exist without the aid of flagella (Abeygunawardana *et al.*, 1990; Abeygunawardana *et al.*, 1991b; Amann *et al.*, 1996). For example, darting, sliding, swarming, and social gliding motility in *Myxococcus xanthus* and twitching motility in *P. aeruginosa* and *Neisseria gonorrhoeae* (Henrichs *et al.*, 1972; Amano *et al.*, 1996; Amellal *et al.*, 1998), which are mediated by type IV pilus (Strom and Lory, 1993; Wall and Kaiser, 1999).

Social gliding motility is a strategy used by myxobacteria to coordinate gliding of cell group and has many similarities to twitching motility in its absolute dependence on type IV pili (Semmler *et al.*, 1999; Wall and Kaiser, 1999). However, unlike twitching motility, social gliding motility in *M. xanthus* is also dependent on peritrichous carbohydrate-protein fibrils that link cells together and facilitate the cohesive movement of large groups of cells (Spormann, 1999; Shimkets, 1999; Wall and Kaiser, 1999; Kearns and Shimkets, 2001; Wolgemuth *et al.*, 2002). Later works showed that twitching motility appears to be principally a means of colonization over moist surfaces and other forms of complex colonial behaviour, including the formation of biofilms and fruiting bodies (Watson *et al.*, 1996; Shi *et al.*, 1996; Ward and Zusman, 1997; O'toole and Kolter, 1998a) and this occurs by the extension, tethering, and retraction of type IV pili, propelling bacteria across surface (Mattick, 2002).

Biofilm is defined as an assemblage of microbial cells developed on surfaces and enclosed in an extracellular polymeric substance matrix (Donlan, 2002). There are many works related with biofilm development owing to usually they exert a negative effect on host causing disease in humans, animals and plants. The developmental steps in biofilm formation include (i) initial attachment to a surface, followed by (ii) cellular aggregation and formation of microcolonies, and finally (iii) maturation of biofilm via the formation of larger sessile mushroom-shaped multicellular structures encased in an extrapolymeric substance matrix composed of a mixture of components, such as EPS, proteins, nucleic acids, and other

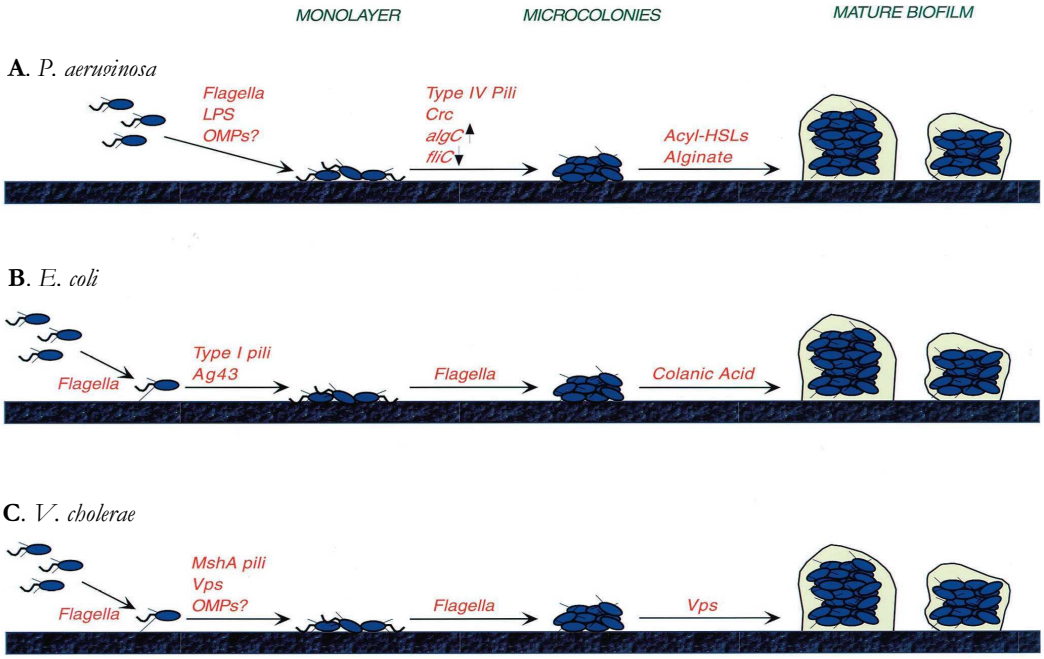

Figure 5.1. Biofilm formation of the best-studied model organisms, *P. aeruginosa, E. coli*, and *V. cholerae* described by Davey and O'toole (2000). (**A**) In *P. aeruginosa*, flagella are required to bring the bacterium into proximity with the surface, and LPS mediates early interactions, with an additional possible role for outer membrane proteins (OMPs). Once bacteria are on the surface in a monolayer, type IV pilus-mediated twitching motility is required for the cells to aggregate into microcolonies. The production of pili is regulated at least in part by nutritional signals via Crc. Documented changes in gene expression at this early stage include upregulation of the alginate biosynthesis genes and downregulation of flagellar synthesis. The production of cell-to-cell signalling molecules (acyl-HSLs) is required for formation of the mature biofilm. Alginate may also play a structural role in this process. (**B**) In *E. coli*, flagellum-mediated swimming is required for both approaching and moving across the surface. Organism-surface interactions require type I pili and the outer membrane protein Ag43. Finally, the EPS known as colanic acid is required for development of the normal *E. coli* biofilm architecture. (**C**) *V. cholerae*, like *E. coli*, utilizes the flagella to approach and spread across the surface. The MshA pili, and possibly one or more unidentified outer membrane proteins, are required for attachment to the surface. This initial surface attachment appears to be stabilized by EPS. Formation of the mature biofilm, with its associated three-dimensional structure, also requires production of EPS. Vps refers to the EPS produced by *V. cholerae*.

substances (Figure 5.1) (Pringle and Fletcher, 1986; Nyvad and Kilian, 1990; Poulsen *et al.*, 1993; O'toole and Kolter, 1998b; Costerton *et al.*, 1999; O'toole *et al.*, 2000; Sutherland, 2001; Stoodley *et al.*, 2002).

It has been reported that strains pertaining to *Pantoea agglomerans* species form biofilm in several environments as for example on stainless steel or on needleless central venous catheter connectors (Murga *et al.*, 2001; Chmielewski and Frank, 2004). Inside *Pantoea* genus, the role of biofilm formation in stewart's wilt disease caused by *Pantoea stewartii* subsp. *stewartii* has been recently studied (Koutsoudis *et al.*, 2006). Specifically, it has been described that the production of the stewartan EPS by *P. stewartii* subsp. *stewartii*, which was regulated via EsaI/EsaR quorum sensing system, was required for its normal biofilm development and pathogen activity. However, reports about biofilm formation by biocontrol agents pertaining to *P. agglomerans* species on plant have not been performed yet.

SEM and TEM observations performed from EPS125 cultures grown in apple juice and apple fruit together with pathogen spores showed the early steps of biofilm development of *P. agglomerans* EPS125. According to latter works, the large amount of PLS interconnecting EPS125 cells observed might be type IV pili, which may be implied in twitching movement of cells and in the formation of microcolonies. Below, the small cell clusters might grow by means of cell division spreading daughter cells upward and outward (Heydorn *et al.*, 2000) and by recruitment of cells from the bulk fluid forming a large number of cell aggregates or symplasmata (Tolker-Nielsen *et al.*, 2000). In addition, in response to surface attachment signal, EPS125 cells probably induce the production of alginate polysaccharide, which surrounds cellular clusters. It is likely that all processes occurring during biofilm formation of *P. agglomerans* EPS125, together with other unknown biocontrol mechanisms, may interfere in germination process of *Penicillium* spores avoiding disease. However, this assumption deduced from results of phenotypic characterization has to be confirmed with genotypic analysis.

To demonstrate which mechanisms are responsible of the biocontrol activity of *P. agglomerans* EPS125 against *P. expansum* in apple fruit, a genetic analysis by means of random mutagenesis with transposons was performed. This technique provides useful information about gene or genes that might be related with biocontrol mechanisms from unknown genomic sequences. For example, Dekkers *et al.* (1998) showed the involvement of a site-specific recombinase in root colonization of the efficient root colonizing biocontrol strain *P. fluorescens* SBW25 using mutagenesis. Nevertheless, sequences disrupted by the transposon insertion might not have a known function or be described in other reports making difficult its study. For this reason, the genetic analysis has to be accompanied by phenotypic studies of mutants impaired in their biocontrol activity. With this in mind, a phenotypic and genotypic characterization of mutants impaired in their biocontrol activity was performed by using minitransposon GUS (mTn5SS*gusA40*). Minitransposon GUS contains *Sm/Spc* genes conferring resistance to spectinomycin and streptomycin antibiotics, respectively, and a promoterless reporter gene GUS (Wilson *et al.*, 1995). Owing to absence of GUS activity in plants and many bacteria, this transposon constitutes a powerful tool to study the induction of genes up-regulated *in vivo* or in *ex vivo* assays (Aguilar *et al.*, 2002). Nevertheless, the use of mutagenesis with transposon technique for studies of biocontrol mechanisms involves some limitations besides those stated in the above section. For example, once transformant colonies containing transposon inserted randomly in its genome have been obtained by conjugation, it has to decide what mutants will be selected, either all mutants, including both auxotrophic and non auxotrophic mutants, or non auxotrophic mutants. In case of choosing all mutants, it has to take into account that the possible absence of biocontrol activity of some mutants may be due to auxotrophy and not to the true lost of its biocontrol mechanism. In addition, the study of all transformants involves an enormous number of mutants to be submitted to the screening program. This implies on one hand, a time-consuming effort for the researcher depending on the screening system used, and on the other hand, a considerable risk during genetic understanding as it may select mutants with

uninteresting or difficult to analyse genetic information. For these reasons, considering the risk of discard mutants that have biocontrol activity impaired being closely related with its auxotrophy, we decided to submit to the screening program only non auxotrophic mutants.

Another limitation of the mutagenesis technique is that it cannot rule out pleiotropic effects resulting from the disruption of a specific gene. For instance, it has been reported that certain extent enzymes for alginate biosynthesis are recruited from other metabolic processes, such as phosphomannomutase-phosphoglucomutase from lipopolysaccharide production (Goldberg *et al.*, 1993). Therefore, the inactivation of the production of one of these enzymes may result in the lost or variation of several metabolism pathways making difficult the study of its biological function and, specifically, masking which metabolic pathway really takes part in biocontrol activity. Sometimes, when more than one mechanism is involved it is not possible to reach the total loss of biocontrol inhibition. For example, a work performed by Schmidt *et al.* (2001) did not achieve to knock out antifungal activity of *E. herbicola* JII/E4 completely by transposon mutagenesis. They suggested that more than one antibiotic may contribute to the antagonistic activity towards *Eutypa lata* II, as well as the presence of multiple copies of the gene cassette responsible for antibiotic synthesis may exist. Hence, mutagenesis with transposons results in an inadequate technique when several biocontrol mechanisms are involved. However, in spite of these limitations, mutagenesis with transposons is the best option to elucidate which biocontrol mechanisms are employed by any BCA of unknown DNA genomic sequence.

Once the collection of mutants was obtained, it had to develop an adequate screening program to choose mutants with the property under study altered. The screening program used in this work was developed, and the appropriate BCA and pathogen concentrations of application were adjusted by means of dose-response assays. Specifically, when apple wounds were treated with 50 µl of EPS125 suspension adjusted to 10^8 ufc/ml and inoculated with 10 µl of pathogen suspension adjusted to $5x10^4$ conidia/ml, more than 90% of *P. expansum* infections were controlled by strain EPS125 (Chapter I). Therefore, these concentrations of application of BCA and pathogen on apple fruit were suitable to perform screening programs of mutants. Using these doses we ensure that mutants impaired in their biocontrol activity due to transposon insertion will be correctly selected. Likewise, from group of 4032 mutants obtained by mutagenesis with minitransposon GUS, m40, m439, m622, m1210, m2002, m2126, m4015 mutants were chosen to accomplish phenotypic and genotypic studies about their impaired ability in disease inhibition.

First of all, the seven mutants were phenotipically characterized to observe relevant differences between mutants and parental strain in relation to several aspects such as colony morphology, growth potential in LB broth, growth on apple fruit, expression of disrupted genes, production of signal molecules of quorum sensing communication system, production of exopolysaccharides and cell interactions with *P. expansum*. The results of the phenotypic characterization of 7 mutants are summarised in Table 5.1.

The transposon insertion did not affect colony morphology of mutants when these were grown on ABM minimal medium, as well as it did not significantly alter their growth curves using LB broth as nutrient source. In addition, population levels reached by the parental strain and selected mutants on apple fruit were similar at both temperatures of incubation, 13 and 23 ºC, showing no significant differences among them. Therefore, it seems that the mutation event has not impinged on multiplication ability of mutants either, when they use LB broth as nutrient source or when they develop on apple wounds. However, the fact that all mutants are able to use the same nutrient sources than strain EPS125 for growth does not imply that they follow the same ecological fitness as the mutation may have altered other features related with colonization ability, production of substances or biofilm development.

Table 4.1. Phenotypic characterization of seven mutants selected in comparison to parental strain EPS125.

Mutant	Morph.	Growth	13ºC	23ºC	GUS LB	GUS apple	QS Ec	QS Cv	IR	SEM	TEM ut	TEM ns
m40	+	+	+	+	↑	↑↑	+	+	+	+	n	n
m439	+	+	+	+	+	+	+	+	+	+	n	n
m622	+	+	+	+	+	+	+	+	+	+	n	n
m1210	+	+	+	+	↑	↑↑	+	+	+	+	n	n
m2002	+	+	+	+	+	+	+	+	+	+	n	n
m2126	+	+	+	+	↑	↓↓	+	+	+	V	+	+a
m4015	+	+	+	+	+	↓	+	+	+	V	n	+a

Morph., colony morphology; Growth, growth in LB broth; 13ºC, multiplication on apple at 13ºC; 23 ºC, multiplication on apple at 23ºC; GUS LB, induction of GUS gene using LB broth; GUS apple, induction of GUS gene using apple juice; QS Ec, detection of HSL using *E. coli* (gfp) bacterial sensor system; QS Cv, detection of HSL using *Chromobacterium violaceum* bacterial sensor system; IR, detection of the 1060 cm-1 band using infrared spectroscopy; SEM, observation through SEM; TEM ut, observation of ultrathin sections through TEM; TEM ns, observation of negative stained samples.
+, same result than *P. agglomerans* EPS125; ↑↑, very high GUS expression level; ↑, high GUS expression level; ↓, low GUS expression level; ↓↓, very low GUS expression level; V, phenotype different of parental strain with aberrant PLS and without microcolonies; n, not analysed; a, low amount of symplasmata in comparison with parental strain.

The use of reporter genes to study gene expression in several conditions is a widespread practice and it has shown to be very useful in the identification of bacterial up-regulated genes in plant–microbe interactions. For example, Aguilar *et al.* (2002) identified 12 genes up-regulated in the pathogen *Erwinia chrysanthemi* during the infection process in chicory leaves using the same minitransposon employed in this work. Unlike of this study, our goal was the analysis of expression of disrupted genes that may be involved in biocontrol mechanism used by strain EPS125. For this purpose, all mutants were grown in *in vitro* conditions using LB broth and sterile apple juice as nutrient sources. Results showed that mutants m40 and m1210 increased the expression of disrupted genes when were grown in apple juice. Therefore, growth medium composition is an important factor that controls the expression of specific genes that may be implied in biocontrol mechanism of parental strain. Nonetheless, the change and increase of expression was not so obvious in m2126 and m4015 mutants, in which induction of impaired genes was triggered in both growth media showing a variable and inconsistent result. Hence, unlike of previous mutants, the influence of medium composition cannot be correlated with the change in gene expression in these mutants. Finally, the absence of GUS activity in m439, m622 and m2002 mutants in the two growth conditions may be due to conditions tested were not appropriate to induce the expression of impaired genes. However, it might be a consequence of the transposon polarity. The promoterless reporter gene GUS can be expressed only when it is inserted in an appropriate orientation relative to bacterial regulatory sequences. Therefore, it may be that minitransposon GUS has situated in the opposite direction to transcription direction making impossible the visualization of GUS activity in these mutants. In conclusion, this assay shows an evident relationship between the up-regulation of some genes with the growth conditions and medium composition; and a possible indirect relation between the up-regulation of genes responsible of biocontrol activity of parental strain and the composition of apple juice growth medium. However, to study the induction of genes related with biocontrol mechanisms employed by strain EPS125 in the control of *P. expansum* disease it has to develop an assay more approximate to *in vivo* conditions, such as on apple wounds and in contact with pathogen spores.

According to the results stated above about the possible implication of alginate production in the inhibition of infections caused by *P. expansum*, it was interesting to analyse whether mutants impaired in biocontrol activity had lose the alginate producing ability. To achieve this purpose, IR analysis and TEM observations were performed on mutants and parental strain cultures grown in apple juice. The IR spectra of all mutants shared the same IR absorption spectrum showed by parental strain maintaining a band associated with the presence of alginate exopolysaccharide. On the contrary, the alginate band was not detected in cultures of strain EPS125, and m2126 and m4015 mutants grown in LB broth. In addition, as it was

observed in parental strain, the characteristic polisaccharic sheath surrounding cellular aggregates was observed in TEM micrographs of ultrathin sections stained with ruthenium red of m2126 culture grown in apple juice, but not in cultures grown in LB broth. Nonetheless, although quantitative studies were not performed, the frequency and the size of these cellular clusters or symplasmata seemed to be less frequent than in parental strain. Hence, these results strengthen the theory previously stated that cellular aggregates are immersed in alginate polysaccharide and its production depends on the nutrient source used for growth. Moreover, the alginate producing activity was not interrupted by the transposon insertion in any of the mutants analysed. However, because of IR and TEM techniques provide qualitative information, the possibility that mutation event might alter a regulation system of alginate production cannot be discarded.

Another aspect considered in our study was the observation of interaction between EPS125 and mutant cells with *P. expansum* spores through SEM. In this way, mutants m40, m439, m622, m1210 and m2002 that were incubated with pathogen spores in inserts of Millicell® tissue culture plates containing apple juice as nutrient source, showed a phenotype similar to parental strain. Pathogen spores still have not germinated and bacterial cells were interconnected by a large amount of PLS forming cellular agglomerations that surrounded conidia. However, unlike of these phenotypes, m2126 and m4015 mutants did not form the large characteristic cellular aggregates of parental strain resting as continuous sheet on the substratum, and PLS were almost undetectable. Nonetheless, the presence of PLS was less evident in m4015 mutant than in m2126, and an unidentified substance surrounding m4015 cells closely linked to filter surface was observed. In addition, as in the case of other mutants, pathogen spores did not germinate yet when were incubated with m2126 and m4015 mutant cells. This situation totally changed when mutant cells were coinoculated with *P. expansum* conidia in apple wounds and incubated for 5 days. Then, mutants were unable to inhibit the infection process as conidia germinated forming a dense mycelium with a large amount of conidiophores invading apple wound surface. Even though mutants have lose biocontrol activity against *P. expansum*, they exert a remaining action against the pathogen delaying its germination process, as when conidia are inoculated alone on apple wounds, they completely invade the wound surface after 24 h of incubation. Therefore, the delay of the germination process due to the presence of mutant cells might imply that mutants have regulation systems of genes related with biocontrol mechanisms altered, as well as other processes or biocontrol mechanism are likely to be involved in inhibition.

Unlike SEM images of cultures of EPS125 and mutants, PLS were not clearly observed in apple wounds. This is hardly surprising as pili usually are expressed in the first stages of biofilm formation which achieves its structural maturity after 10 days of incubation (Stoodley *et al.*, 1999; Heydorn *et al.*, 2000).

According to other reports, PLS of strain EPS125 may be responsible of processes such as attachment and movement of cells during the EPS125 biofilm development. Similarly to the results obtained by O'toole and Kolter (1998a) wherein mutants defective in the biogenesis of type IV pili were unable to form the characteristic microcolonies of early biofilm development, m2126 and m4015 mutants impaired in PLS form cellular sheet densely packed without microcolonies essential for biofilm formation. All these observations seem to point towards PLS are directly related with EPS125 biofilm development. However, the absence of PLS in m2126 and m4015 mutants was not corroborated by another microscopical technique less stringent than SEM. Negative staining micrographs of mutants grown in apple juice show long PLS linking cells identical to parental strain. Therefore, it is possible that mutation has affected some point of assembly or disassembly processes of PLS making weaker and more susceptible to their removal during SEM processing. Concomitantly, because of their weakness, PLS might be unable to accomplish their biological function during biofilm development and disease suppression.

The continuous cellular sheets without differentiation observed in SEM micrographs of m2126 and m4015 mutants, also were very similar to the biofilm produced by a double mutant of *P. aeruginosa* PAO1 defective in production of quorum-sensing molecules acylhomoserine lactones (acyl-HSLs) (Davies *et al.*, 1998). Owing to this similarity, it was decided to analyse whether EPS125 and mutants synthesized and had disrupted the production of quorum-sensing signal molecules through two bacterial sensor systems. Results obtained showed that all mutant strains of EPS125 were able to produce HSLs (3-oxo acyl-HSLs, 3-hydroxy acyl-HSLs, and alkanoyl-HSLs with chain lengths ranging from C4 to C12), which bears out that mutants maintain the acyl-HSLs production activity. However other molecules different from acyl-HSLs have been described in cell-to-cell communication of *P. agglomerans* strains. Specifically, a work performed by Cha *et al.* (1998) showed that most erwinias analysed produced N-(3-oxo-hexanoyl)-L-HSL, as well as they produced quite different signals including 3-hydroxy forms and active compounds that chromatographed with proprieties different from their standards. In addition, it has been described that *E. agglomerans* strains also produce cyclo (ΔAla-L-Val) quorum sensing signal molecule, a type of diketopiperazines (DKPs) (Holden *et al.*, 1998). Therefore, we cannot discard that mutations have impaired the production of quorum sensing signal molecules as several signals of quorum sensing may be involved during biofilm formation by *P. agglomerans* EPS125.

Once mutants were phenotipically studied and characterized, we proceeded to their genotypic analysis. Firstly, the absence of pCAM140 plasmid into mutant cells was confirmed showing that minitransposon was inserted in genomic DNA of mutants. Secondly, the number of transposon insertions in selected mutants was determined by means of southern blot. Except for m2196, m3152, m3427 and m3891 mutants, other mutants showed a single hybridized fragment as it was desirable. This analysis also confirmed that there were not siblings and that all mutants had transposon inserted in different points of their genomic DNA. Afterwards, we performed the sequencing of transposon insertion sites of m40, m439, m622, m1210, m2002, m2126 and m4015 mutants. However, due to singularity of parental strain, it was a difficult task and only was achieved for m40, m2002, m2126 and m4015 mutants.

Analysis of nucleotide sequences of m2002 and partially m4015 mutants indicates that *P. agglomerans* EPS125 has homologous sequences to IS1222 insertion sequences described in strains from the same and closely related species. However, nucleotide sequences of m40, m2126 and partially m4015 mutants do not reveal any similarity with nucleotide sequences within GenBank database. Nonetheless, these share similarity to dihydrodipicolinate synthase (DHDPS), luciferase family protein and lysine/ornithine N-monooxygenase protein sequences, respectively.

The protein sequence deduced from transposon flanking region in m40 mutant showed a 35 % identity with aminoacid sequence of DHDPS proteins from different microorganisms and this was the same value found by Blickling *et al.* (1997) between bacterial DHDPS of *Escherichia coli* and *Nicotiana sylvestris*. While the plant DHDPS sequences are highly homologous to each other, the bacterial sequences have been shown to be strongly divergent and only residues important for maintaining the tertiary structure (Mirwaldt *et al.*, 1995), the formation of the active site (Blickling *et al.*, 1997) and the inhibitor binding site have been conserved in all sequences. A sequence alignment performed by Blickling *et al.* (1997) of all known plant and bacterial sequences showed that the overall similarity between the enzymes from the two kingdoms were comparable to the diversity between any two bacterial sequences. Therefore, because of high diversity found among bacterial DHDPS sequences, we consider that the protein sequence deduced from m40 sequence shows high similarity with bacterial DHDPS proteins. DHDPS is a key enzyme of the *meso*-diaminopimelate (*meso*-DAP) biosynthetic pathway in bacteria and plants. Specifically, DHDPS encoded by *dapA* gene catalyses the condensation of pyruvate and L-aspartic acid semialdehyde forming dihydrodipicolinate intermediate (Rodionov *et al.*, 2003). *meso*-DAP is an important metabolite in higher plants, most algae and prokaryotes. It

is the direct precursor of lysine, a member of the aspartic acid family of amino acids that also includes methionine, threonine and isoleucine. Moreover, DAP is an essential constituent of the peptidoglycan layer in many bacterial cell walls and its absence leads to cell death (Cirillo *et al.*, 1994).

Because of m40 mutant is not lysine auxotroph and has the biosynthetic way of DAP unaltered being able to grow in minimal defined media; it has three transposon insertions into its genome according to southern analysis; and the DNA sequence flanking to transposon did not show similarity with any DNA sequence deposited in the GenBank database, place this mutant as an unsuitable candidate to be consider for further studies.

Lysine biosynthesis genes of *E.coli* have been found scattered along the chromosome. Orthologs of these genes were identified in bacterial genomes including enterobacteria, *Pasteurellaceae*, *Vibrionaceae* and *Shewanella oneidensis*, wherein these genes also were stand separately. However, Gram-positive bacteria from the *Bacillus* and *Clostridium* group have lysine biosynthesis genes either as single genes or within a LBS gene cluster, potentially forming an operon (Rodionov *et al.*, 2003). It is suspected that *P. agglomerans* EPS125 has these lysine biosynthesis genes dispersedly arranged because any similitude with the nucleotide sequence of 1000 b next to "*dapA*" gene was found. For this reason, this peculiar sequence of unknown function was selected in the Chapter I to develop a possible DNA molecular marker of strain EPS125.

DNA sequence of transposon flanking regions in m2126 mutant did not show similarity with any nucleotide sequence deposited in GenBank database. However, the protein sequence deduced from 48 to 753 b and from 876 to 1382 b of DNA sequence had large identity with proteins deduced from ORF9 (47%) and ORF10 (33%) of *Comamonas* sp. strain NCIMB 9872, respectively. These open reading frames were located in a cluster grouped in four possible transcriptional units (*orf11-orf10-orf9*)-(*cpnE-cpnD-orf6-cpnC*)-(*cpnR-cpnB-cpnA*)-(*orf3-orf4* [partial 3' end]). The *cpnABCDE* genes encode enzymes for the conversion of cyclopentanol to glutaric acid, which provides to strain NCIMB 9872 the ability to grow on 0.1% cyclopentanol as sole carbon source. However, the specific role of *orf9*, which encodes a putative alkanal monooxygenase, and *orf10*, which has not a known function associated, in this metabolic pathway has not been solved (Iwaki *et al.*, 2002). Therefore, there is not any based reason to believe that disrupted sequence from m2126 mutant was related with the cyclopentanol metabolism. In addition, the deduced protein from 48 to 753 b of the nucleotide sequence of m2126 mutant, showed greater identity values with bacterial luciferase family proteins of several microorganisms. Bacterial luciferase family proteins are TIM barrel enzymes that include F420-dependent N5, N10-methylenetetrahydromethanopterin reductases (Mer), alkanesulfonate monoxygenases (SsuD) and bacterial FMN-dependent luciferase (LuxAB) (Eichhorn *et al.*, 2002; Aufhammer *et al.*, 2004). These three enzymes are structurally related in spite of the low sequence identity among them and their different biological function (Eichhorn and Leisinger, 2001).

Mer is involved in CO_2 reduction to methane in methanogenic archaea and, specifically, it catalyses the reduction of N5, N10-methylenetetrahydromethanopterin with reduced coenzyme F420 to N5-methyltetrahydromethanopterin and oxidized coenzyme F420 (Aufhammer *et al.*, 2004). Mer is found in methanogenic and sulfate reducing archaea (Ma and Thauer, 1990; Tebrommelstroet *et al.*, 1990; Ma *et al.*, 1991; Vaupel and Thauer, 1995; Shima *et al.*, 2002), and hence, it is unlikely that disrupted sequence by transposon insertion of m2126 mutant is related to Mer protein.

SsuD is a monoxygenase that catalyzes the conversion of alkanesulfonates to the corresponding aldehyde and sulfite. Under conditions of sulphate or cysteine starvation, this enzyme allows *E. coli* to use a wide range of alkanesulfonates as sulfur sources for growth (Eichhorn *et al.*, 2002). Inorganic sulfur needed for the biosynthesis of sulfur-containing compounds is poorly represented in some environments. Hence, bacteria including *P. agglomerans* EPS125 may develop alternative sources for obtaining this element as for example, leading to the

synthesis of SsuD protein. However, owing to SsuD has been absolutely required for the desulfonation of methanesulfonate and L-cysteate (Eichhorn *et al.*, 1997) and, the growth ability of m2126 mutant on apple wound did not show significant differences to parental strain, overall points out that the impaired gene of m2126 might be unconnected with SsuD function.

Finally, the heterodimeric luciferase enzyme encoded by *luxA* and *luxB* genes of the *lux* operon (*luxICDABEG*), catalyses the oxidation of aldehyde and reduced flavin mononucleotide to form a long-chain fatty acid, water and flavin mononucleotide. Simultaneously, the liberation of excess free energy, evident as blue-green light, occurs resulting in the bioluminescent phenotype of microorganisms such as the marine bacterium *Vibrio fischeri* (Eberhard *et al.*, 1981; Engebrecht and Silverman, 1984). The luminescence *lux* operon is regulated by the transcriptional activator *Lux*R and acyl-homoserine lactone autoinducers molecules in a population density-responsive manner named quorum sensing. Quorum sensing is described as a regulatory mechanism of gene expression in response to population density in gram-negative bacteria. Genes controlled are involved in different activities including luminescence, the production of extracellular enzymes, plasmid transfer, swarming, antibiotic synthesis, protein secretion, capsular polysaccharide synthesis, and biofilm formation (Engebrecht and Silverman, 1984; Piper *et al.*, 1993; Fuqua *et al.*, 1994; Eberl *et al.*, 1996; Chapon-Herve *et al.*, 1997; von Bodman *et al.*, 1998). For example, *Pantoea stewartii* subsp. *stewartii* emploies quorum sensing regulation in the production of the EPS virulence factor called stewartan (Whitehead *et al.*, 2001) and *Erwinia carotovora* in the production of carbapenem antibiotic and exoenzymes (Swift *et al.*, 1993).

There are several microorganisms described with different cell-to-cell signaling systems such as *P. aeruginosa*, which has the *las*R-*lasI* and *rhl*R-*rhlI* systems that regulate more than 40 genes (Hassett *et al.*, 1999; Whiteley *et al.*, 1999; Pesci and Iglewski, 1999). These two regulation systems have been involved in biofilm formation of *P. aeruginosa*, and at sufficient population densities these signals reach concentrations required for activation of genes involved in biofilm differentiation (Davies *et al.*, 1998). However, as it has stated above, atypical biofilm architecture, thinner and with cells more densely packed was produced by mutants unable to produce both signals. Specifically, this effect on the biofilm structural complexity has been related to possible deficiencies in twitching motility (De Kievit *et al.*, 2001) and reduced extracellular polysaccharide (EPS) production (Shih and Huang, 2002).

Therefore, according to sequencing information and microscopic observations showed in the present work, mutant m2126 may have genes related with PLS production that might be regulated by quorum sensing system impaired, influencing the formation of the typical biofilm structure of the parental strain. In addition, other specific functions regulated by quorum sensing may have been affected such as alginate production. Because of only a qualitative determination of alginate has been performed by means of IR, we cannot discard that mutation has altered its production. In addition, it has shown that no differences of HSL detection were observed using two bacterial sensor systems between m2126 mutant and than *P. agglomerans* EPS125, and therefore it seems that mutation has not affected its production. However, it is unknown if this strain produces other signalling molecules, such as HSL-variants or diketopiperazines described in *E. agglomerans* (Holden *et al.*, 1998), which may have been affected by the mutation. Hence, we cannot determine what point has altered the transposon insertion; however, we have many evidences that relate the lack of biocontrol activity with the formation of aberrant PLS, which might be a consequence of a quorum sensing regulation system altered by transposon insertion. Nonetheless, we have to bear in mind that results obtained may be related with pleiotropic effects of the mutation that affect, amongst other things, biofilm formation pathways and other unknown functions that might be involved in biocontrol activity of parental strain.

The protein sequence deduced from transposon flanking regions in m4015 mutant showed similarity with Lysine/ornithine N-monooxygenases from different microorganisms. This enzyme is encoded by *iucD* gene, which is included in the aerobactin cluster (*iucABCD-iutA*)

implied in biosynthesis of the aerobactin siderophore (*iucABCD*) and of the receptor protein (*iutA*) (Challis, 2005). Iron is an essential nutrient for microorganisms because it is a cofactor for several enzymatic processes including pathogenic processes. However, the availability of iron is very low because at neutral and alkaline pH, ferric iron forms insoluble and polymeric complexes that cannot be assimilated by microorganisms. A common strategy used by many pathogenic and saprophytic microorganisms to tackle this problem is the biosynthesis and excretion of high-affinity iron chelators of low molecular weight known as siderophores. Siderophores are exported from the cell and chelate ferric iron from the environment, the iron–siderophore complex is then transported into the cell via membrane-associated ATP-dependent transport system that often exhibits high substrate selectivity (Drechsel and Jung, 1998; Koster, 2001).

It is possible that transposon insertion produced in m4015 mutant has altered the production of either aerobactin siderophores or membrane receptor protein, affecting the acquisition of external iron by *P. agglomerans* EPS125. Therefore, a great amount of iron rests available for *P. expansum* conidia, which require a large input of iron for germination process (Charlang *et al.*, 1981). The production of siderophores such as enterobactin, hydroxamate, and ferrioxamine by members of enterobacterial genera including *Erwinia, Pantoea, Enterobacter, Hafnia* and *Ewingella* has been demonstrated in several reports (Berner *et al.*, 1988; Reissbrodt and Rabsch, 1988; Reissbrodt *et al.*, 1990; Berner and Winkelman, 1990; Berner *et al.*, 1991; Matzanke *et al.*, 1991; Koebnik *et al.*, 1993; Feistner and Ishimaru, 1996; Deiss *et al.*, 1998).

Owing to the growth ability of m4015 mutant on apple wound was the same than parental strain being not affected by the possible iron lack suggests that *P. agglomerans* EPS125 may have other siderophore systems of iron acquisition. It is not surprising because it has been reported bacteria that are able to produce different siderophores. For example, *P. syringae* pv. *tomato* DC 3000 has all genes required for biosynthesis of pyoverdin and yersiniabactin siderophores (Meyer, 2000) and *P. syringae* pv. *syringae* B728a has gene cluster for pyoverdin biosynthesis and genes closely related to biosynthetic genes of achromobactin (Franza *et al.*, 2005). Nevertheless, in the present work, the presence of iron acquisition systems in *P. agglomerans* EPS125 has not been studied.

Besides siderophore production, recently it has been demonstrated that limited iron availability results in an increase in gonococcal pilin antigenic variation, DNA transformation, DNA repair and pili production (Serkin and Seifert, 2000; Tomich and Mohr, 2004). Specifically, iron limitation was shown to induce expression of type IV pilus of *Moraxella catarrhalis* (Luke *et al.*, 2004) and pilus of *Burkholderia cenocepacia* (Tomich and Mohr, 2004). This fact supports the speculation that bacteria are prepared to colonize host surfaces with low-iron availability by upregulating pilus expression.

According to these studies, the transposon insertion produced in the putative *iucD* gene of m4015 mutant might produce two consequences, an inactivation of iron acquisition system using aerobactin siderophore, and an alteration of PLS production, which is supported by SEM observations. This bears out the suspicion that PLS play an important role in biocontrol of *P. expansum* by *P. agglomerans* EPS125, and adds a new biocontrol mechanism related with iron competition.

Several works have reported that aerobactin genes, from plasmid or chromosome, are associated with a repetitive sequence identified as IS1. It has been suggested that the presence of these insertion sequences could promote transposition as well as deletion of the aerobactin genes (Cornelis and Saedler, 1980; Kleckner, 1981) and could provide a site for recombination between DNA containing the aerobactin genes and other DNA elements which have IS1 (Kleckner, 1981). Hence, this function also could be attributed to IS1222 insertion sequence placed near to the putative *iucD* gene found in m4015 mutant.

Genetic analysis performed in the present work on m4015 and m2002 mutants provided an interesting result. The nucleotide sequences obtained from both mutants showed high identity (90%) with IS1222 insertion sequences from close related species such as *E. coli*, *Salmonella*

enterica, *Enterobacter agglomerans*, *Rahnella aquatilis* and *Yersinia pestis*. This agrees with a study performed by Steibl and Lewecke (1995), wherein IS1222 is limited to a group of related bacterial strains among *Enterobacteriaceae*. According to sequence alignments and structural features, IS1222 has been included in a subclass within IS3 family of bacterial IS, together with IS407, IS476 and ISR1 (Fiandt *et al*., 1972; Prere *et al*., 1990; Wood *et al*., 1991). These type of genetic elements are very common in bacterial genomes. In particular, the genome of *P. syringae* pv. *tomato* DC3000 has 24 ORFs interrupted by an IS element or phage proteins (Buell *et al*., 2003). It has been suggested that IS elements and transposons contribute to genetic flexibility and environmental adaptation of microorganisms by means of rearrangements or deletions. For example, the production of the slime substance PIA by *Staphylococcus epidermidis* could be turned on and off by means of insertion sequence elements (Ziebuhr *et al*., 2000).

In spite of IS1222 sequence of m2002 and m4015 mutants were identical; we suspect that transposon was inserted in different points of genome due to southern patterns and phenotypic features of both mutants were different. Therefore, it is possible that *P. agglomerans* EPS125 has several IS1222 copies such as *Enterobacter agglomerans* 339, which contains three copies on the *nif* plasmid pEA9 and one on its chromosome. Owing to nucleotide sequence obtained from m2002 mutant only showed homology with this IS sequence, it is unknown what biological function related with biocontrol activity of parental strain has been impaired by transposon insertion. In addition, it is possible that we cannot determine what function may be involved because, as described Jensen *et al.* (2004), the IS elements found in *P. syringae* pv. *tomato* DC3000 are located more frequently within the regions containing genes of unknown functions.

Therefore, having into account that few sequences deposited in GenBank database are related with genes responsible of biocontrol activity, mutagenesis with transposons technique has provided interesting information about possible mechanism used by *P. agglomerans* EPS125 against *P. expansum* different from antibiosis. In this way, different genes were identified and the corresponding sequences were classified according to their possible biocontrol function in the present work as: (i) unknown function genes, (ii) genes implied in quorum sensing regulation system, (iii) genes involved in biosynthesis and excretion of aerobactin siderophores, and (iv) insertion sequences related with transposition events. Nonetheless, mutagenesis with transposons has not clarified or resolved completely what are the biocontrol mechanism used by *P. agglomerans* EPS125 against *P. expansum* because pleiotropic effects might occur and other biocontrol mechanisms related with impaired genes of mutants that were not sequenced may be implied. This finding is quite usual when working with BCAs, for example Ellis (1998) was not able to identify a single and specific common locus for biocontrol-deficient mutants, which were obtained by mutagenesis with transposons from biocontrol agent *Pseudomonas fluorescens* SBW25.

To demonstrate that impaired biocontrol phenotypes showed by the four sequenced mutants were due to transposon insertion, each mutation has to revert recovering partial or totally the biocontrol activity of *P. agglomerans* EPS125. Usually, it is achieved by complementation of the mutation placing in mutant cells the original and functional gene from parental strain, which might be obtained from a genomic library of the strain under study. However, the construction of a genomic library for strain EPS125 was attempted with different commercial systems without success. The impossibility to generate a genomic library together with the fact that only 4 of 7 mutants were able to be sequenced, seem to be a consequence of the singularity of the parental strain.

Results from the present work together with other showed by Bonaterra *et al.* (2003) demonstrated that the antagonistic activity of *P. agglomerans* EPS125 is not based on the production of antibiotics, which is a very important criterion in selecting biological agents for protection of food products (Smilanick and Denisarrue, 1992; Wisniewski and Wilson, 1992; Sobiczewski *et al*., 1996). In this way, overall data and observations contributed in this work provide valuable information that has not been presented before about implication of biofilm

formation and other mechanism in biocontrol of *P. expansum* by *P. agglomerans* EPS125 in apple fruit.

Biofilm formation not only is an important adaptive strategy of bacteria to environmental conditions in several milieus (Emmert and Handelsman, 1999; Davey and O'toole, 2000), but also it has been related with biocontrol mechanisms employed by some BCAs. In the case of the biocontrol agent *Pseudomonas fluorescens*, it has been thought that it forms biofilms on plant roots. However, the relationship between biofilm formation and biocontrol has not been confirmed (O'toole and Kolter, 1998b; Bianciotto *et al.*, 2001). In addition, numerous biocontrol mechanisms have been outlined for *Bacillus subtilis* strains, wherein the secretion of the lipopeptide antimicrobial agent surfactin has been also included. Nevertheless, as in the case above, the specific role of biofilm formation on plant roots has not been characterized yet (Chanway, 2002).

The development of a biofilm over apple tissue surface implies beneficial properties to strain EPS125. For example, water-filled channels that enclosed cell clusters enhance oxygen and nutrient availability and remove potentially toxic metabolites; EPS provides a structural support for the biofilm, concentrates growth essential components and enhances the water retention preventing desiccation processes; attached cells are less likely to be displaced by physical processes; antagonist cells are better able to withstand competition from newly invading organisms; they are more resistant to predation, during initial stages of colonization individual cells may benefit from nutritional opportunities that result from concerted activity of the entire colony; and it provides an ideal niche for cell communication via quorum sensing (Debeer *et al.*, 1994; Costerton, 1995; Roberts, 1996; Wolfaardt *et al.*, 1998; Roberts *et al.*, 1999; Hausner and Wuertz, 1999; Davey and O'toole, 2000; Sutherland, 2001; Donlan and Costerton, 2002; Stephens, 2002).

Observations performed in this work allow the development of a hypothetical model for biofilm formation of *P. agglomerans* EPS125, wherein inhibition of germination of pathogen spores is also involved (Figure 5.2). Firstly, antagonist cells may attach to the apple surface using a variety of cell surface components such as PLS and alginate. As it has been described in some yeast biocontrol agents, these structures may provide a colonization advantage to strain EPS125 being more competitive than pathogen (Andrews *et al.*, 1994). PLS, or rather type IV pili, of strain EPS125 can bind to a variety of surfaces including inert surfaces, other bacterial cells, and pathogen spores producing twitching motility by means of pili assembly and retraction. This movement brings cells into close alignment forming cellular clusters, in where pathogen conidia are included. Similarly, initial adhesion on apple surface is followed by the production of extracellular polysaccharide alginate by EPS125 cells, which only is induced under certain growth conditions. This behaviour has been also reported in *Pseudomonas* spp. and *Pantoea* spp strains, which produce EPS after attachment on cut surfaces (Morton *et al.*, 1998). Alginate may play an important role in adhesion of EPS125 cells to apple surface, determining biofilm structure, protection of cells from external stress, concentration of essential nutrients and components for biofilm growth, and in this case, it surrounds pathogen spores forming an unfavourable milieu for its germination process. In addition, EPS125 cells may be able to take up iron from apple fruit by means of siderophores slowing down iron availability to pathogen cells, and avoiding its germination process. Therefore, EPS125 biofilm architecture based on microcolonies with nutrient and oxygen gradients present acceptable conditions for growth of anaerobic facultative bacteria such as EPS125. However, this microenvironment brings down the availability of compounds such as water, iron or oxygen that are indispensable for germination of *P. expansum* conidia (Charlang *et al.*, 1981; Gqaleni *et al.*, 1996). Therefore, biofilm formed by strain EPS125 cells seems to condition the tissue-surface milieu by their physiological activity inhibiting the pathogen development and protecting them from the adhesion of extraneous organisms. In addition, it has been proved that strain EPS125 forms HSL molecules, which are implied in the regulation via quorum sensing of specific target genes that may be responsible of biofilm

differentiation and biocontrol activity. Specifically, it may control the formation of PLS, which is an important factor for the normal development of biofilms, and other unknown biocontrol mechanism used by *P. agglomerans* EPS125 against *P. expansum*.

Finally, the preventive but not curative action of the antagonist strain against several fungal pathogens (Bonaterra *et al.*, 2003; Francés *et al.*, 2006), as well as the fact that inhibition of germination of pathogen spores only was achieved when both microorganisms are in direct cell-to-cell contact (Bonaterra *et al.*, 2003) strengthens the hypothesis of biofilm development with associated biocontrol activity, as pathogen spores only were able to germinate when they did not found a physical barrier that keeps the necessary signals for conidia germination. Because of complexity of these studies, there are few works where the relationship between biofilm formation and biocontrol mechanism is established and, to our knowledge, any of them have been performed in postharvest context. Therefore, overall results stated in the present work provide interesting information to postharvest biocontrol field about possible implication of biofilm formation of *P. agglomerans* EPS125 with its biocontrol activity.

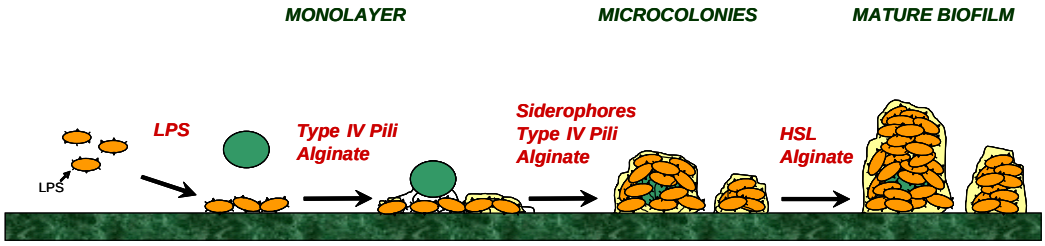

Figure 5.2. Hypothetical model of biofilm formation of *P. agglomerans* EPS125 (orange cells) and biocontrol mechanisms used by strain EPS125 against *P. expansum* (green cells). EPS125 cells may attach to apple wound surface by means of lipopolysaccharides (LPS). Once bacteria are on the surface in a monolayer, type IV pilus may mediate twitching motility to aggregate cells into microcolonies, where pathogen conidia may be included. The nutrients composition of apple tissue and attachment signal trigger production of alginate polysaccharides providing microcolonies formation and increasing the attachment to the surface. EPS125 cells synthesize siderophores to take up iron from milieu. Pathogen spores are surrounded of cell-alginate layer that retains all specific signals for its germination process. The production of cell-to-cell signalling molecules (acyl-HSLs) may be required for formation of the EPS125 mature biofilm.

In conclusion, our findings point out that biocontrol mechanisms used by the effective biological control agent *P. agglomerans* EPS125 against *P. expansum* are directly related with its ability of biofilm formation on apple fruit. On one hand, biofilm provides to the antagonist strain an appropriate milieu for its growth and a physical structure that allows to withstand environment stresses. On the other hand, EPS125 biofilm inhibits the blue mould postharvest infection depriving *P. expansum* spores of indispensable compounds such as water, oxygen or iron that trigger the germination process. Therefore, according to the fact that strain EPS125 does not produce antifungal metabolites, it constitutes an interesting product to develop commercially. However, to validate these results, mutations have to be complemented with respective unaltered genes from parental strain that could be obtained from EPS125 genomic library recovering biocontrol activity. Nevertheless, it has not been achieved yet because genomic DNA of strain EPS125 has intrinsic attributes that makes difficult its manipulation. In addition, these assumptions have to be accompanied by studies directed to determine the specific role that plays each component in biocontrol activity of strain EPS125 such as interference with production of PLS, alginate, siderophore or HSL by means of physical methods; target mutagenesis of biosynthetic genes of these compounds; or observation of biofilm formation through confocal scanning laser microscope.

Conclusions

1. According to morphological and biochemical tests, strain EPS125 pertain to *Pantoea agglomerans* (*Enterobacter agglomerans-Erwinia herbicola*) species. This strain did not present ice nucleation activity causing frost damage on plants, and it is harmless for plants according to the absence of hypersensitive response on tobacco.

2. The study of effectiveness of the biocontrol agent *P. agglomerans* EPS125 against *Penicillium expansum* by means of dose-response assays showed that this strain was highly effective against *P. expansum* in apple fruit having a median effective dose from $2.7x10^5$ to $7x10^5$ cfu/ml and a ratio of 101 and 25 EPS125 cells to inactivate one pathogen spore according to the hyperbolic saturation model.

3. *P. agglomerans* EPS125 was singular being the only one strain among 14 representative strains studied unable to use tween 40 as carbon source and able to metabolize mono-methyl succynate; as well as to contain 14:1 w5c, SIF1 and 15:0 ISO 3OH fatty acids. In addition, strain EPS125 showed a characteristic macrorestriction fragment length polymorphism (MRFLP) pattern of 13 fragments (302, 270, 259, 253, 197, 179, 165, 160, 140, 110, 81 and 71 Kb) by digestion of DNA with *XbaI*.

4. Two DNA molecular markers, 125.2 and 125.3, were developed for monitoring of *P. agglomerans* EPS125. Each molecular marker was semispecific for its detection by PCR when they were used separately, because it amplified five strains with the 125.2 primer set and a strain in case of the 125.3 primer set from an overall of 267 strains. Nevertheless, strain EPS125 was the only one that showed amplification signal with both molecular markers suggesting that the combined use of the two primer sets in a multiplex PCR reaction for the specific detection of *P. agglomerans* EPS125 would be successful. Nowadays, a real time PCR using 125.3 design has been developed and proved to be semispecific for strain EPS125.

5. A transformation protocol by electroporation was developed for *P. agglomerans* EPS125 being the better conditions growth in LB agar, cleaning with 1mM HEPES pH6.8 supplemented with 10 % glycerol solution, and transformation of electrocompetent cells with 1 μg of DNA at 2.5 kv/cm, 200 Ω and 25 μF.

6. *P. agglomerans* EPS125 is able to produce biofilm of with multicellular aggregate structures or symplasmata surrounded by an alginate saccharic layer, which is produced under certain growth conditions (apple juice); and a large amount of LPS structures interconnecting cells,

which may be implied in twitching movement of EPS125 cells and in the assemblage of cells for formation of microcolonies.

7. Production of antifungal compounds by *P. agglomerans* EPS125, as main biocontrol mechanism to inhibit *P. expansum*, was discarded using *in vitro* assays with concentrated cell-free culture filtrates grown in apple juice. In addition, competition for nutrients has shown not to be the main biocontrol mechanisms according to interaction experiments with a semipermeable filter that allowed the metabolite interchange. Direct interaction arise as an important biocontrol mechanism as cell-to-cell contact between biocontrol and pathogen was needed to achieve the inhibition of conidia germination.

8. A collection of 4032 non auxotrophic mutants was obtained by mutagenesis with minitransposon GUS (mTn5SS*gusA40*). Seven mutants completely defective in biocontrol of *P. expansum* in apple fruit were chosen. No differences with regard to colony morphology; growth potential; multiplication ability on apple fruit; and production of signal molecules of quorum sensing and alginate were observed between selected mutants and parental strain. However, mutants m2126 and m4015 did not form microcolonies resting as a continuous sheet on the substratum and their LPS were almost undetectable. The genetic analysis was solely accomplished for m40, m2002, m2126 and m4015 mutants.

9. The genomic sequence flanked to minitransposon of m40 mutant was sequenced and the deduced hypothetical protein showed a 35% of identity with aminoacid sequence of dihydrodipicolinate synthase (DHDPS) proteins of several bacteria. DHDPS is a key enzyme of the biosynthetic pathway of *meso*-diaminopimelate (*meso*-DAP) in bacteria and plants, which is the direct precursor of lysine and an essential constituent of the peptidoglycan layer of bacteria and its absence leads to cell death. Owing to m40 mutant was able to grow in minimal defined media, had three transposon insertions; and the DNA sequence flanking to transposon did not show similarity with any DNA sequence, discard this mutant for further studies.

10. The hypothetical proteins deduced from nucleotide sequence of m2126 mutant, 876-1392 b and 48-753 b, shared a large similarity with a hypothetical protein of *Comamonas* sp. (33 %) of unknown funtion and with bacterial luciferase family proteins of several microorganisms (55 %) respectively. It is likely that m2126 mutant has damaged the regulation system via quorum sensing affecting the normal production of LPS by m2126 mutant and its consequent biofilm formation.

11. The protein sequence deduced from m4015 mutant showed 41 % of identity with lysine/ornithine N-monooxygenases which are implied in aerobactin siderophore biosynthesis. It is suspected that mutation produced in m4015 mutant may be altered the ability to acquire external iron, resting large amounts available for *P. expansum* which is indispensable for germination of *P. expansum* spores. In addition, the iron limitation that m4015 mutant may sustain due to transposon insertion might alter the production of LPS producing the abnormal biofilm showed by m4015 mutant.

12. Nucleotide sequences of m2002 and m4015 mutants showed 90 % of identity with IS1222 insertion sequences, which contribute to genetic flexibility and environmental adaptation of microorganisms through rearrangements or deletions. Although both sequences were identical, it is likely that transposon was inserted in different points of genomic DNA of two mutants due to their southern patterns and phenotypic features were not the same. Therefore, *P. agglomerans* EPS125 may have several IS1222 copies into genome.

13. Biofilm formed by *P. agglomerans* EPS125 may be involved in the inhibition of germination of pathogen spores. Specifically, several compounds such as LPS, alginate, siderophores and gene regulation via quorum sensing seem to be important for its biofilm development, as well as they may be implied in the biocontrol activity of strain EPS125.

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ANNEXES

ANNEX I. Media, buffers and reagents

ABM with 10% mannitol

NH_4Cl	2 g
$MgSO_4 \cdot 7H_2O$	0.3 g
KCl	0.15 g
$CaCl_2 \cdot 2H_2O$	0.01 g
$FeSO_4 \cdot 7H_2O$	0.0025 g
K_2HPO_4	3 g
$KH_2PO_4 \cdot 2H_2O$	1.15 g
Agar	15 g
Distilled water	1000 ml

Sterilization by autoclaving at 121ºC for 20 min. Add 2 g of manitol sterilized by filtration.

Biolog universal growth agar (BUG™)

BUG ™ Agar	57 g
Distilled water	1000 mL

Adjust pH to pH 7.3 ± 0.1. Sterilization by autoclaving at 121ºC for 20 min.

Buffered peptone water

Na_2HPO_4	7.1 g
K_2HPO_4	2.72 g
Peptone	1 g
Distilled water	1000 ml

Adjust pH to 7.2-7.4. Sterilization by autoclaving at 121ºC for 20 min.

Chitinase test medium

Colloidal chitin*	1.5 g
K_2HPO_4	2.7 g
$KHPO_4$	0.3 g
$MgSO_4 \bullet {}_7H_2O$	0.7 g
NaCl	0.5 g
KCl	0.5 g
Yeast extract	0.13 g
Agar	20 g
Distilled water	1000 mL

Adjust pH to 7.0-7.2. Sterilization by autoclaving at 121ºC for 20 min.

* Colloidal chitin was prepared as Rodríguez-kabana *et al.* (1983) described.

Denaturalization buffer

NaOH	0.5 M
NaCl	1.5 M

EDTA

EDTA 2•H_2O	93.05 g
Distilled water	400 mL

Adjust pH to 8 with NaOH. Sterilization by autoclaving at 121ºC for 20 min.

GUS stock (20 mg/ml)

X-GlcA*	20 mg
Phosphate tampon 0.1 M pH 7.0	0.5 ml
Distilled sterile water	0.4 ml
EDTA 0.5 M pH 8.0	2 µl

*5-Bromo-4-chloro-3-indolylβ-D-glucuronide; Sigma, Saint Louis, USA

HEPES buffer 10X

HEPES	9.53 g
Sodium acetate	5.44 g
EDTA	1.49 g
Distilled water	1000 ml

Adjust pH to 8.0. Sterilization by autoclaving at 121ºC for 20 min.

Indol acetic medium (IAA)

Tryptone	10 g
NaCl	10 g
Yeast extract	5 g
Agar	15 g
Distilled water	900 ml

Sterilization by autoclaving at 120ºC for 20 min. Add 100 ml of 50 mM L-tryptophan sterilized by filtration.

Luria Bertani agar medium (LB) (Sambrook and Russell, 2001)

Triptone	10 g
NaCl	10 g
Yeast extract	5 g
Agar	15 g
Distilled water	1000 mL

Sterilization by autoclaving at 121ºC for 20 min.

Luria Bertani broth (LB) (Sambrook and Russell, 2001)

Triptona	10 g
NaCl	10 g
Yeast extract	5 g
Distilled water	1000 mL

Sterilization by autoclaving at 121ºC for 20 min.

Lysis buffer

Tris HCl pH 8.3	10 mM
KCl	50 mM
Tween 20	0.1 % p/v

Sterilization by autoclaving at 121ºC for 20 min.

MG/L media

LB	500 ml
Mannitol	10 g
Glutamic acid	2.32 g
KH_2PO_4	0.5 g
NaCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Biotin	2.0 µg

Bring up to 1 L with distilled water. Sterilization by autoclaving at 121ºC for 20 min.

Neutralization buffer

NaCl	1.5 M
Tris HCl	1 M

Adjust pH to 7.4.

Nutritional agar medium (NA)

Yeast extract	3 g
Glucose	2.5 g
NaCl	5 g
Peptone	5 g
Agar	15 g
Distilled water	1000 mL

Adjust pH to 7.3 ± 0.1. Sterilization by autoclaving at 121ºC for 20 min.

O/F medium

Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Glucose	10 g
Bromotimol blue	60 mg
Agar	5 g

Sterilization by autoclaving at 121ºC for 20 min.

Phosphate buffer

Na_2HPO_4	7.098 g
KH_2PO_4	2.723 g
Distilled water	1000 mL

Sterilization by autoclaving at 121ºC for 20 min.

Physiological buffered water (AFT)

NaCl	8 g
$NaH_2PO_4 \cdot 2H_2O$	0.4 g
$Na_2HPO_4 \cdot 12H_2O$	2.7 g
Distilled water	1000 ml

Adjust pH to 7.2-7.4. Sterilization by autoclaving at 121ºC for 20 min.

Potato dextrose agar (PDA)

Potato dextrose agar	39 g
Distilled water	1000 mL

Adjust pH to 5.6 ± 0.2. Sterilization by autoclaving at 121ºC for 20 min.

Prehybridization solution

20X SSC	12.5 ml
100X Denhardt's	2.5 ml
10% SDS	2.5 ml
Distilled water	Bring up to 50 ml

Reactive 1 (NaOH-MetOH solution)

NaOH	30 g
Methanol (HPLC)	100 mL
Distilled water	100 mL

Reactive 2 (HCl-MetOH solution)

HCl 6N	325 mL
Methanol (HPLC)	275 mL

Reactive 3

Hexane:methyl-tert-butyl ether 1:1

Reactive 4 (saturated NaCl)

NaCl (saturated solution)

Saline solution

NaCl	8.5 g
Distilled water	1000 mL

Sterilization by autoclaving at 121ºC for 20 min.

Salkowski reagent

$FeCl_3$ (2%)	0.5 M
$HClO_4$	35 %

Semidefined medium (SDM)

Yeast extract	4 g
$CaCl_2 \cdot 2H_2O$	0.04 g
$FeSO_4 \cdot 7H_2O$	0.04 g
$MgSO_4 \cdot 7H_2O$	0.0024 g
K_2HPO_4	4 g
KH_2PO_4	4 g
Na_2HPO_4	5.73 g
NH_4Cl	0.2 g
$(NH_4)_2SO_4$	1.2 g
Agar	15 g
Distilled water	1000 ml

Sterilization by autoclaving at 121ºC for 20 min. Add 10g of glucose sterilized by filtration.

SEP buffer

EDTA	25 mM
Na_2HPO_4	25 mM
NaH_2PO_4	25 mM
Sucrose	0.3 M

Adjust pH to 8.0 with NaOH. Sterilization by autoclaving at 121ºC for 20 min.

Sequencing reactive

Sodium acetate 3M pH 4.6	1.5 mL
Ethanol 95%	31.25 mL
Distilled water	14.25 mL

SOB

Bactotrip	5 g
Yeast extract	1.25 g
Distilled water	244 ml

Sterilization by autoclaving at 121ºC for 20 min. Add 0.5 ml of 5M NaCl, 0.63 ml of 1M KCl, 2.5 ml of 1M $MgCl_2$, and 2.5 ml of 1M $MgSO_4$ solutions sterilized by filtration.

SOC

Bactotrip	5 g
Yeast extract	1.25 g
NaCl	0.125 g
250mM KCl	2.5 ml
Distilled water	Adjust volume to 250 ml

Sterilization by autoclaving at 121ºC for 20 min. Add 5ml of 1M glucose and 1.25 ml of 2M $MgCl_2$ solutions sterilized by filtration.

SSC 20X

NaCl	3 M
NaCitrate	0.3 M
Adjust pH to 7.0	

TB buffer

PIPES 0.1M pH6.7	50 ml
$MnCl_2$ 1M	27.5 ml
$CaCl_2$ 1M	7.5 ml
KCl 1M	125 ml
Distilled water	290 ml

Sterilization by autoclaving at 121ºC for 20 min.

TEN

Tris 1M pH8.0	4 ml
EDTA 0.5 M	8 ml
NaCl 5M	12 ml
Distilled water	Bring up to 400 ml

Tris-Acetate-EDTA (TAE) buffer 50X

Tris base (Sigma, N5125)	121 g
Glacial acetic acid	28.55 mL
EDTA 0.5M pH8.0 (sterile)	50 mL

Dissolve in 350 mL of milli•Q water. Adjust pH to 7.5-7.8 with concentrate HCl. Bring up to 500 mL with milli•Q water.

Tryptone soy B agar (TSBA)

Tryptone soy B agar	40 g
Distilled water	1000 mL

Sterilization by autoclaving at 121ºC for 20 min.

Wash solution I (2X SSC, 0.1% SDS)

SSC 20X	10 ml
SDS 10 %	1 ml
Distilled water	Bring up to 100 ml

Wash solution II (1X SSC, 0.1% SDS)

SSC 20X	5 ml
SDS 10 %	1 ml
Distilled water	Bring up to 100 ml

Wash solution III (0.1X SSC, 0.1% SDS)

SSC 20X	0.5 ml
SDS 10 %	1 ml
Distilled water	Bring up to 100 ml

ANNEX II. DNA sequences.

DNA sequences obtained from pBS-40, pBS-2002, pBS-2126 and pBS-4015 of 1814 b, 1652 b, 1473 b and 3619 b of length respectively.
The 125.2 molecular marker was developed from nucleotide sequence of pBS-m2126, and the 125.3 molecular marker was developed from nucleotide sequence of pBS-m40. Arrows show hybridization position and synthesis direction (5'→3') of primers of 125.2 (red) and 125.3 (green) designs. Rectangles show hybridization position of probes 125.2 (red) and 125.3 (green) designs.

m40

1 ACTCACTATA GGCGAATTGG AGCTCCACCG CGGTGGCGGC CGCTCTAGAA NNANAGAAAT ATNNCCTGCC TCCATGATAT CGACATCCGC

91 TTCAANAANA GCAGAGTAAT AATTATTGAC GAGATCTTCA TCGAAATTCC AGTTGGTATA GTAGCCACCA TCACGGAAAG TGCAATCCAA

181 CAAAACTGCA TTAGAATTAG TCATCATTGT TCATCACTTT TGTATAGCAA CATGCGCATC ACGCATGTTG CTGGATGTGA CATCAGTAGC

271 AAGAAAACAG TATTTTTAGA TTAATACTGT ACCGGCTCCG GTCGGGAATC GAACTGATAG AATTTATCCA TTACTGCCCA TTCGGTAGGG

361 CCATCATTCA TAAATACCCG CTGTAGCAGC TCTCCATGAA CATAATCATC AAACGCTTTA ACTTTTGGAT CTAATGTCAG CGCATGAGTG

451 AACTCACGTG AAGGTGAGAA TTCATCAATA GTTTCCATAT ACATAAACAA CATGTTGGGT TGCATAAAGA AAATTTGCAT CGTTTTAATC

541 CCAATCGCCG CCAGCGCACC GTCTCCGGCA TGAATTTCTG GAATTTGATT CAGATGCATT TCCCGATAAA TTTCGACGGC TTTACGGTTA

631 CGCAATTCGA TAGTAAGTCC CCAACTTTTC ATAATTTCCT CTTTTATTGG CAGATGTTAT CTAGAACAGA AATAATACGG TCAATTTCTT

721 TATAGGTCAC AACGTCGGGC ACATGATCAA CCGGTTCCCG AACGCGTGGA TGAGAAATAA GACCTTTGCG ATATAAAATG TGTTTCGAAA

811 GATGCTGATA GCAATCAATA CTATTCATCA TTTGGCACAT CATGTAAGAA AGAGGGTAAG CCAGTTTTTC CGCCGATTTT ATATCCCCTG

901 CCTGTAAATA ACTCCAGATT TTCACCACAT ATTTAACGCA ATCGGTAGCA GGTATGGAAC CATACAGTCC ACGGAAATAG CTGTCGATAA

991 TCATCATACC GCCATCACCC TCGAAGATCC GAGCCTGATT ATCGGTGGCA AGCTGTAATT CAGTGTGCTT TGGAGGCAGC GGCGACGATT

1081 CAGGTTTAAA CAGTATTCTG TCGGCGCCAA ACTCGTGTAA AAGATCCGCC TGCATTTTTA TCGTCATGGG TGTTTTAGCA TAACTTTTTG

1171 CATGGTGAAT CATGACTGGG ATATGAAGTT GCTCAATAAC ATCCCGGTaA TATTTATGCA TACGCTGTTC GGTTAGCGGT GAAGTGGATG

1261 GGgTGCATTA ATAATACGGC ATCCACGCCA GACTCTTGGG CATACAAAGA ATATTCGATA GCGTTTTTAG TACTTTCACC TCCCGAACTC

1351 ACGACAACGT GACCTTTAGG CTTGCCATAT TTCACGGCCA TATCCGTAAT GTTCTCGCGT TCGCGGCTAT TCATTCTCAT CAGTTCAGAA

1441 ACCTGACCAA TGACGATGCC ATCGGCACCG ACTTCATAGC AATAATCAAC TTGCTTAGCA AAGTCATCTT CATGGATATC AAAATTCTCA

1531 TCCCAAGGAG TTAATATAAC GGGGAGAACA CCTTTAACAT TTGTATAAGA AGACATATTT AACCCTTAAT TATATTGTTA GTGATGCGCC

1621 TAGCTACCTC TGGAGCCAGT TTAAAACCTC CGCCGGAATA GCCTGTCAAA ATAGATAATG AAGCCAAGCC TTCAATCTTT TCATCAGCAC

1711 CGACACCTTC AGCGCTGAAT AACAATCNNN GCTGACCATG AGCCACATTC TTTTGCCNGA NGACCCNNTT AATCTTTTCT GCCCCATCAT

1801 ATTAGTCACG TCGC

m2002

1 TTCAGAAACA ATTGTTTTAT CTGCTTTGTT AGACACAGAG TTAGTAATGT CGGTTTGACT ACCTTCTAAT TTTCTGTTTC TCCAGCCCGC

91 TGCAAATTCA GATGGTGTCT GGTAATTCAG CGACGAATGT GGACGGCACT CGTTATAATC CAGCCGCCAG TCATTAATGA TTTTCCTGGC

181 GTGAACAATA TCGCTGAACC AGTGCTCATT CAGGCATTAA TCACGAAAGC GTCCGTTAAA ACTCTCAATA AATCCGTTCT GCGTTGGCCT

271 ACCCGGCTGG ATTAAGCGCA ACTCCACGCC ATGCTCATAA GCCCACTGGT CAAGTGCTCT GCAGGTAAAC TCCGGCCCCT AGTCAGTTCT

361 TATAGTGGCC GGATAGCCGC GAAACAGCGC AATGCTGTCC AGAATACGCG TGACCTGCAC GCCTGAAATC CCGAAGGCAA CAGTGGCCGT

451 CAAGCATTCC TTTGTGAATT CATCAACACA GGTAAGGCAC TTGATCCTGC GACAGGTGGC CAGTGCGTCC ATGACGAAAT TCATCGACCA

541 GGTGCACCAT CGGGCGGAGC AGCGGCAGAC GTTCTGTTGC CAGCCCTCCC GCTTCTGGCC TGTCGTCAGT ACTTTCGCCC AGTAGCCACC

631 TGAAGCGCCT CCTTATCCAG CATGGCTTCA GCAAGCAGCT TCCTGAGTCT GGCGTTCTCT TCCTCAAGTG ACTTCAGGCG CTTAACCTCA

721 GGCACCTCCN TACCGCCATA CTTCTTACGT CATGTGTAAA ACGTGGCATC TGAAATGGCG TGCTTACGGC AGAGCTTACG GGCACAAACC

811 CCGGCTTCGG CCTCGCAGAG AATACTGATG ATCTGTTCGT CGGAAAAACG CTTCTTCATG GGGATGTCCT CATGTGGCTT ATAAAGATAT

901 TACTAACATC GCGGTGTTAA TCAACGGGGA GCAGGTCATG AATTGNATAT TACCCTGACA GGATGAATCT TAATAAGCAG ACATCGGTGT

991 TACTAGCTTC ATTCTTTCTA AGCAATCCTC ATTGTGGAAT CCTCGCGCAT CACTGCTTAA GGGTGAAAAA CATTGGTGAA CACAGGTAAT

1081 ACATGCAATT TATTTAATGT TAACGCTAAT AATAACTTAT GTTAATAGTT AGAAATCTAT TTTTTATGAG TCAAGTAAAT TTTAGTGATT

1171 CAGTGCATTG GTTAACAAAA TATTACGATT TCGCGTAGGT TATAGGTACA GGTGGATAAG GGATATCGCA TAAAAAAACC ATTAAGTATT

1261 GTTTTTTCAA GTGGTTAACT AAGTTTTCTG GCCTTTGTGG TTTGAAGGTG ATTAATACGT TAGAAATCAA TAAATAAATT TTTATATCGA

1351 AATAAATGAA AAAAGACGAC AAATGTTTCA ATGTTGTTAC TCATGCTCGC TTATAATTCT TAAATAAAAT TATAAAAGTA GAGTTTTGTT

1441 TCGATGTTGC TTTTGACATG TAAATAAATT GTTTACATAA GGTAAATTAA TAACTAATAA TGACGCTCGA ATTGATTACA TCAGTAAAGC

1531 TTATGCACGG GTACTGTCAT CTACTAGCAA ATTAAACGCC TATTGGTTAT GGCGACTTAA GCCTAGCTGG TCTCAAAGCT CGTTTCATAT

1621 TGTAAATCTA ATGNGAATTA ANGAATGAGA GT

m2126

1 GNGCGAATTG GAGCTCCaCC GCGGTGGCGG CCGCTCTAGA aNNaNttGGA tttcttgcct CCCAAACTAA ACGCATAACC CTGGgNaNTA

91 CCGGTATGGt GttACCTCTG CGGGACCCGA TTATATTGGC CAAGCAGGCA GCCTCTGTGG ACCAGCTAAG TGGTGGGCgc cttCtgCTGG

181 GATTGTCCAG tGGGGATCGC GCTGTAGAAT ATCCTGCGTT TGGAGCCGAT TATGATAACC GTGCCGAACG CTATCGGGAG GCGTTTGGTC

271 TGATTAAAAC CGTCAGTGAG ACGTCCTTCC CGGTGGCTGA AACGGCGTTT TACGGAAGGC TTTCAGGCAA CCTTGATCTT ATTCCTAAAC

361 CTTTTAAATC CCGCATTCCG ATGATTGTGG TAGGGCGGGC GCGGCAGGAC CTGAGCTGGA TAGCTGAAGA AAGCGACGGA TGGATATGGC

451 ATCTGAGTGA CTTCTCcACG CTCCCggaat tGCTTGAATT CTGGCGTGGC GATTACgATG ACAATCGtTT CagACCTTAT GGcTATGCCa

541 CCTTTTTTGA cCTCgATGCT AAcCCTGAcg CCCCGCTTCg CAgattGATG AATGGcATcA CTGTCGGCCG CAATGCGCTG ATAAGCTATG

631 GAAGGAGCAC NNACCCGCCA GGGGGTAAAC CACGTTGCGT TAAATCTGAA ACCGCTGACA CGGCCCGCAG CCGAGGTGAT GGCTGAAATG

721 GCAGAATTCG TGTACCCGAG TTTCCTTCTG AATAAACGCA GGACAATAAA TGAACAGTTC ATTTCATTCT TCGGTGCAGA GCCTGATTAG

811 TTTTTGTGAA ACCTGGTCAG GAAATGCTTG CTCAGACAAT AGCCAATGGC TGGACCCCTC GGTCAGATTA GTCAGCAGTC ATCGTGGAAC

901 AGCTTTCCGG CCGTGAAAAT GTCACCGCCC TTTTGCAAAA TGACTTTGCT GACCTGGATA ACGTATCCGT TACGCTGACA AACACCGTTG

991 AGCGCCGGAA CGATAAAGAT TACGTCGCCA GTGCATACCT GCACGGGCAG GCTTCGCGCA AAGGTTCCGG ACGTCATAAC CAGGTGCGGT

1081 TTGGGGGGAC GGTCGTGATG AGGGCAGGCA CAGATGAGTC CCAACCTGTT ATCCATACTC TTCACCTGCA AATaACCTGG ACTTCAGGTG

1171 CAAGGCAGTT GTTATCGGGA TGGCTGCTAC CGGCTGAAGA ATCCGGCTGG CATCAGGGAT TTCCGCAAGT CGCCATCATC AGTGAACTTG

1261 ACGCCCCCTG GCATATTGTG CCAGAAAACC ACCGTGATAC GAGTGAAGAA CAGGGAGTCA TCGATACCTG GTATCGCTAT GCGTGGGCGC

1351 TGGACCAGGC AGATTTCAAA CTTTTCAGCA CAGTGCTTTA GTGAAAATGC TGCGGGTAAT TTTACCCCCC CTGGGCTNCA TATCAGGGAG

1441 GCGGAATATT ATTGCCGCAA TGAAAGCCTT

m4015

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1    CGACTCACTA TAGGGCGAAT TGGAGCTCCA CCGCGGTGGC GGCCGCTCTA GAACTAGTTT CAGAAACAAT TGTTTTATCT GCTTTGTTAG
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361  GCTCATAAGC CCACTGGTCA AGTGCTCTGC AGGTAAACTC CGGCCCCTAG TCAGTTCTTA TAGTGGCCGG ATAGCCGCGA AACAGCGCAA
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541  TAAGGCACTT GATCCTGCGA CAGGTGGCCA GTGCGTCCAT GACGAAATTC ATCGACCAGG TGCACCATCG GGCGGAGCAG CGGCAGACGT
631  TCTGTTGCCA GCCCTCCCGC TTCTGGCCTG TCGTCAGTAC TTTCGCCCAG TAGCCACCTG AAGCGCCTCC TTATCCAGCA TGGCTTCAGC
721  AAGCAGCTTC CTGAGTCTGG CGTTCTCTTC CTCAAGTGAC TTCAGGCGCT TAACCTCAGG CACCTCCATA CCGCCATACT TCTTACGTCA
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2791 GACTCGTTAA GAAAAAGAAT TATGGATCTT CATGCTGATA CCAATTACTC AGTGGTCGAT GGGGATTTAA TTACTGAATT ATATAAAAAA
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3151 AATCGCCAGA TGGTGGGTAA AATATATTTA CCCGGAATGT CGGAAGGCCA TCATGGTTTG TCGGCTACAC TGCTTTCTTT ATTACCGATA
3241 AGGGCTCAGG AAATTGTTCG GGATGTGGTT AACTCAGCCT GTTTCAAAGC CTCTTCCACA ACCACCGAGG TTCTGAATGT CAATTAAGCA
3331 GAACTACGAT GTGGTGGTAA TAGGCGCAGG TATTCTTGGG GCGGCTACAG CACATCAGTT AACCTGTAGA GGCTTAAAAA CAGCCTTATT
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3601 GGCTCAAGAT AATGCCCGG
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