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E. S. D.

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Re: Expediente No. 13-275.266

Solicitud de patente a nombre de **BAYER CROP SCIENCES LP.**

Yo, SILVANA MARIA LOPEZ DÍAZ identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, piso 6, obrando como apoderada de la Sociedad **BAYER CROP SCIENCES LP**, domiciliada en California, Estados Unidos, solicitante de la patente de la referencia, en respuesta al oficio No. 13208 notificado por fijación en lista el 14 de octubre de 2014, relacionado con concepto técnico de fondo, para cuya respuesta fue necesario solicitar un plazo de 30 días hábiles, mediante memorial del día 17 de diciembre de 2014, me permito manifestar ahora lo siguiente:

1. Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial, un nuevo pliego reivindicatorio que consta de un total de 22 reivindicaciones, en donde se han eliminado las reivindicaciones 16 a 22, 28 a 60 y 63, en un esfuerzo por acelerar el trámite.

2. Excepción a la Patentabilidad

El artículo 20, literal (d) de la Dec. 486 establece:

Artículo 20.- No serán patentables:

[...]

d) los métodos terapéuticos o quirúrgicos para el tratamiento humano o animal, así como los métodos de diagnóstico aplicados a los seres humanos o a animales.

Su Despacho ha objetado a las reivindicaciones 53 a 60 por reportar materia que hace referencia a una excepción a la patentabilidad, de acuerdo con lo establecido en el artículo arriba citado. De este modo y teniendo en consideración lo anterior, se informa que dichas reivindicaciones han sido eliminadas del actual pliego reivindicatorio, con lo cual se supera la objeción planteada en este respecto.

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3. Usos y Segundos Usos

El artículo 14 de la Dec. 486 establece lo siguiente:

Artículo 14.- Los Países Miembros otorgarán patentes para las invenciones, sean de producto o de procedimiento, en todos los campos de la tecnología, siempre que sean nuevas, tengan nivel inventivo y sean susceptibles de aplicación industrial.

Su Despacho ha indicado que las reivindicaciones 28 a 52 y 63, reportan usos de la composición reclamada, lo cual no se considera materia patentable de acuerdo con lo establecido en el artículo arriba citado. Siendo así las cosas, se informa que las reivindicaciones citadas han sido eliminadas del actual pliego reivindicatorio.

4. Claridad

El artículo 30 de la Dec. 486 de la Comunidad Andina establece:

Artículo 30.- Las reivindicaciones definirán la materia que se desea proteger mediante la patente. Deben ser claras y concisas y estar enteramente sustentadas por la descripción.

4.1 Su Despacho ha objetado los términos “por lo menos”, “uno o más” y “aproximadamente”, por ser ambiguos y no permitir determinar el verdadero alcance de la invención que se desea proteger, ante lo cual se informa que dichos términos, han sido eliminados de las nuevas reivindicaciones 1 a 22.

4.2 Su Despacho ha objetado a la reivindicación 1, por reportar “una combinación sinérgica”, lo cual se considera un resultado a alcanzar y adicionalmente, la composición no está debidamente definida. En dicho respecto se informa, que se ha eliminado el término “sinérgica” y así mismo, se han definido correctamente los posibles compuestos que pueden hacer parte de la composición y la proporción que debe existir entre los dos compuestos activos dentro del sistema. De este modo, se considera que se ha respondido debidamente a la objeción.

4.3 Por último su Despacho señala, que las reivindicaciones referentes a la composición, no hacen referencia a los componentes ni a las proporciones y/o concentraciones en la que éstos se deben encontrar en dicha composición, ante lo cual informamos que las nuevas reivindicaciones 1 a 22, partiendo

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específicamente de la reivindicación 1, reclama debidamente la composición, tal y como se mencionó previamente.

De este modo se considera que se ha respondido a todas y cada una de las objeciones por falta de claridad, razón por la cual se afirma que el nuevo pliego reivindicatorio que se presenta en esta oportunidad, cumple a cabalidad con este requisito.

5. Descripción

Su Despacho indica que el capítulo descriptivo de la presente solicitud no es lo suficientemente claro, para que una persona del oficio versada en la materia, esté en la capacidad de reproducir la presente invención. En dicho respecto manifestamos nuestro completo desacuerdo, dado que en este capítulo hay una descripción general de los lipopéptidos emepleados, siendo estos las iturinas, surfactinas y fengicidas, específicamente en los párrafos [0034] a [0036]. Así mismo, el método para cultivar *Bacillus subtilis* QST713, para producir el producto de fermentación y así generar un extracto de lipopéptidos crudos, está ilustrado en el ejemplo 1 de este capítulo descriptivo y en referencia a los productos comerciales que contienen dicho producto de fermentación de *Bacillus subtilis* QST713, está a su vez descrito en el ejemplo 2.

En lo que concierne con las estructuras químicas de las iturinas, surfactinas y fengicidas, se tiene que éstas ya eran conocidas en el momento de publicar por primera vez la presente solicitud. Ejemplo de ello, es el estudio de Ongena et al., "*Bacillus Lipopeptides: Versatile Weapons for Plant Disease Biocontrol*" Trends in Microbiology, 16(3):115-125, (2007) en donde se describe las estructuras químicas de cada uno de dichos grupos de compuestos (ver figura 1, Anexo).

De este modo se considera que, una persona del oficio versada en la materia que tuviera acceso al contenido de la presente solicitud y al saber de la presencia de compuestos como iturina surfactina y fengicida, los cuales ya eran conocidos en el momento de presentar esta solicitud, ya tendrían presente cuál y como es la estructura química de los mismos.

Con base en lo anteriores argumentos, solicitamos amablemente, considerar por superada esta objeción.

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6. Observaciones con respecto a la falta de Unidad de Invención

El artículo 25 de la Dec. 486, establece:

Artículo 25.- La solicitud de patente sólo podrá comprender una invención o un grupo de invenciones relacionadas entre sí, de manera que conformen un único concepto inventivo.

El señor Examinador señala la presencia de seis grupos inventivos diferentes en la presente solicitud. En dicho respecto mi mandante manifiesta su desacuerdo, en primer lugar porque la presente invención sí es nueva e inventiva, tal y como se demostrará a continuación en las siguientes secciones, por tanto, el argumento del Examinador que acá se expone, carece de toda validez.

Pese a lo anterior y con el ánimo de acelerar el trámite y evitar futuras objeciones del mismo tipo, se han incluido reivindicaciones dependientes que reportan que el fungicida poliénico es natamicina y derivados del mismo y el lipopéptido es un compuesto del tipo iturina o fengicida.

Con base en lo anterior, se le solicita amablemente a este Despacho, tener en cuenta el nuevo pliego reivindicatorio que se presenta en esta oportunidad, así como también los argumentos que se exponen en la misma, con el fin de verificar lo anteriormente afirmado.

7. Observaciones con respecto a la falta de Novedad

De acuerdo al artículo 16 de la Dec. 486, se determina:

Artículo 16: Una invención se considerará nueva cuando no esté comprendida en el estado de la técnica.

"El estado de la técnica comprenderá todo lo que haya sido accesible al público por una descripción escrita u oral, utilización, comercialización o cualquier otro medio antes de la fecha de presentación de la solicitud de patente, o en su caso, de la prioridad reconocida.

La novedad de una invención se evalúa a través de la comparación uno a uno de sus características esenciales. De este modo, una invención puede ser objetada por novedad cuando sus características esenciales son exactamente iguales a las de un antecedente que represente el estado de la técnica conocido al momento de

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presentar la solicitud. Por lo tanto en caso que llegue a existir al menos una diferencia en dicha comparación, se afirma que la invención SI cumple con el requisito de novedad.

De acuerdo con lo establecido en la acción oficial pendiente, se tiene que la reivindicación 1 y sus reivindicaciones dependientes, se ven afectadas por falta de novedad, en vista de las enseñanzas del documento D1: "*Combination echinocandin-Polyene Treatment of Murine Mucormycosis*", Ashaf. S Ibrahim et al., 2008. Ante dicha objeción, se presentan a continuación los siguientes argumentos que la desvirtuarán.

Analizando las enseñanzas del documento D1, se tiene que este hace referencia a una combinación de caspofungina y amfotericina B, la cual produce un efecto sinérgico. Sin embargo se tiene que esta referencia no menciona ni sugiere, combinaciones de fungicidas poliénicos seleccionados del grupo que consiste de natamicina, nistatina y derivados de los mismos y, lipopéptidos seleccionados del grupo que consiste de compuestos del tipo iturina, surfactina y fengicina, tal y como se reporta en la nueva reivindicación 1.

De esta manera se tiene que por el simple hecho de que el documento D1, no consigue divulgar todas y cada una de las características técnicas esenciales que definen a la composición reivindicada, ésta continúa siendo novedosa sobre el estado previo de la técnica, tal y como lo establece el artículo arriba citado.

8. Observaciones con respecto a la falta de Nivel Inventivo

El artículo 18 de la Decisión 486 señala lo siguiente:

Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.

Una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

De acuerdo con lo establecido en la acción oficial pendiente, se tiene que la reivindicación 2 y sus dependientes, carecen de nivel inventivo, en vista de la combinación de las enseñanzas de los documentos D2: ES2268774 y D3: "*Nuevos antifúngicos. Presente y futuro*", 1958. Ante dicha afirmación, mi representada presenta a continuación los siguientes argumentos, que desvirtuarán dicha objeción.

Analizando las enseñanzas de los documentos D2 y D3, se tiene que éste último hace referencia a compuestos antifúngicos de nistatina y natamicina (ver tabal 2, D3), mientras que el documento D2 enseña que la bacteria *Bacillus Subtilis* produce iturina y surfactina. A partir de lo anterior, el Examinador afirma que D2 ya divulgaba que la combinación de un fungicida y un lipopéptido de *Bacillus Subtillis* AQ713, genera un efecto técnico sinérgico, por tanto, una persona del oficio versada en la materia, se vería motivada a combinar una nistatina o natamicina con lipopéptidos, con total de expectativa de alcanzar el mismo resultado y/o efecto técnico sinérgico.

Ante dicho análisis, mi mandante se encuentra en sumo desacuerdo, en vista de que D2 enseña una combinación que comprende un lipopéptido de la bacteria *Bacillus Subtillis* AQ713 y una azoxistrobina, la cual resulta en un efecto sinérgico fungicida. Sin embargo y como bien lo sabe una persona del oficio versada en la materia, la azoxistrobina pertenece a la clase de fungicidas de estrobilurina, los cuales inhiben la respiración en las células de los hongos. En contraste, se tiene que la natamicina y la nistatina son fungicidas poliénicos que se enlazan con los esteroides en la membrana celular de los hongos, principalmente a los ergosteroides.

Con base en lo anterior, se tiene que en el momento que se presentó esta solicitud de patente, la persona del oficio versada en la materia, no esperaba obtener un efecto sinérgico a partir de la combinación de un lipopéptido de *Bacillus Subtillis* AQ713 con una nistatina o una natamicina, lo cual es absolutamente razonable, si se tiene en cuenta las diferencias existentes entre las estructuras químicas de éstos compuestos, con respecto a la de la azoxistrobina.

De este modo se desea resaltar, que las composiciones reclamadas en esta solicitud, sí poseen nivel inventivo por el simple hecho de que éstas producen efectos técnicos sinérgicos y sorprendentes en el control del crecimiento de varias especies de hongos, como por ejemplo lo son *Saccharomyces cerevisiae*, *Penicillium*

expansum, *Geotrichum candidum* y *Geotrichum citri-aurantii*. Dicha sinergia fue compraba, mediante los resultados obtenidos a partir de la ecuación de Gowing. En específico, se evidenció sinergia en la combinación fungicida de una natamicina y un extracto crudo de un caldo de fermentado de *Bacillus Subtilis* AQ713, aplicado a un cultivo de *Saccharomyces cerevisiae* (ver ejemplo 1, del capítulo descriptivo de la presente solicitud).

En experimentos similares, se demostró que las combinaciones de natamicina con un extracto crudo de lipopéptidos o con compuestos de tipos iturina, surfactina o fengicina puros, se obtiene un efecto sinérgico al aplicar dicha composición a cultivos de *Penicillium expansum*, *Geotrichum candidum*, and *Geotrichum citri-aurantii* (ver ejemplos 3, 4, 5 y 7 y las tablas 1, 2 y 4). Resultados similares, fueron encontrados a partir de combinaciones de nistatina con un extracto crudo de lipopéptidos de un caldo fermentado de *Bacillus subtilis* QST713 o con compuestos puros del tipo iturina, surfactina y fengicina (ver ejemplos 6, 8 y 10).

Es así como a partir de las composiciones que se presentan en esta oportunidad, se suministra un beneficio inesperado en el control del crecimiento de hongos patógenos, en cantidades relativamente bajas de los agentes activos de la combinación en cuestión.

Siendo así las cosas y como bien lo sabe este Despacho, el hecho de que toda nueva creación posea una ventaja tecnológica y/o efecto técnico inesperado, los cuales no eran antes contemplados en el estado previo de la técnica, se debe considerar como un avance que dicha nueva creación le está aportando al campo técnico y en consecuencia, se debe reconocer la altura inventiva que dicha creación lleva consigo.

De este modo, se han expuesto las razones por las cuales la presente invención sí cumple con el requisito de nivel inventivo, por tanto se le solicita amablemente a este Despacho, tener en consideración el nuevo pliego reivindicatorio que se presenta en esta oportunidad, en conjunto con los argumentos que se exponen en la misma, para que así reconozca por sí mismo lo anteriormente concluido y se proceda con la concesión de la presente solicitud de patente.

9 Concesión de solicitudes equivalentes

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Si bien entendemos y aceptamos que cada país es autónomo en la concesión o negación de los privilegios de patente, queremos resaltar el hecho que el análisis de los requisitos para el otorgamiento de esos derechos es prácticamente universal. La presente invención fue concedida por la Oficina de Patentes de los Estados Unidos: US8623813 (B2).

10. Conclusiones

Habiendo atendido cabalmente las objeciones presentadas por ese despacho, atentamente solicitamos se conceda el privilegio de patente de invención a la solicitud en estudio o si el señor examinador considera que subsisten razones para objetar la presente invención, rogamos expedir otro examen de fondo con los debidos motivos y razones que sustenten su objeción con el fin de que nuestro mandante tenga la posibilidad de exponer sus argumentos con base en la interpretación de los artículos 45 de la Decisión 486 -el cual otorga la facultad al examinador para emitir los exámenes de fondo-, 35 y 59 del Código Contencioso Administrativo que señala que: *"habiéndose dado oportunidad a los interesados para expresar sus opiniones, y con base en las pruebas e informes disponibles, se tomará la decisión que será motivada al menos en forma sumaria si afecta a particulares"* y *"(..) deberá proferirse la decisión definitiva. Esta se motivará en sus aspectos de hecho y de derecho, y en los de conveniencia si es el caso"*, respectivamente (El subrayado es nuestro).

En consecuencia, solicitamos a ese despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional

Señor Superintendente,



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ANEXO 1

- Nuevo capítulo reivindicatorio compuesto por 22 reivindicaciones
- Copia del artículo Ongena et al., "*Bacillus Lipopeptides: Versatile Weapons for Plant Disease Biocontrol*" Trends in Microbiology, 16(3):115-125, (2007).

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REIVINDICACIONES

1. Una composición CARACTERIZADA PORQUE comprende una combinación fungicida de un poliénico seleccionado del grupo que consiste en natamicina, nistatina y derivados de los mismos y un lipopéptido seleccionado del grupo que consiste de compuestos del tipo iturina, compuestos del tipo surfactina y compuestos del tipo fengicina, en donde la relación peso a peso del fungicida poliénico y el lipopéptido es de 500:1 a 1:500.

2. La composición de la reivindicación 1, CARACTERIZADA PORQUE el lipopéptido es un compuesto del tipo iturina y el fungicida poliénico es una natamicina o un derivado del mismo.

3. La composición de la reivindicación 1, CARACTERIZADA PORQUE el lipopéptido es un compuesto de tipo iturina.

4. La composición de la reivindicación 1, CARACTERIZADA PORQUE el lipopéptido es un compuesto de tipo fengicina.

5. La composición de la reivindicación 1, CARACTERIZADA PORQUE el lipopéptido es una surfactina.

6. La composición de la reivindicación 3, CARACTERIZADA PORQUE el compuesto de tipo iturina es una iturina A.

7. La composición de la reivindicación 3, CARACTERIZADA PORQUE el compuesto de tipo iturina es una bacilomicina.

8. La composición de la reivindicación 1, CARACTERIZADA PORQUE el lipopéptido es un compuesto del tipo fengicina y el fungicida poliénico es una natamicina o un derivado del mismo.

9. La composición de la reivindicación 1 CARACTERIZADA PORQUE comprende dos lipopéptidos.

10. La composición de la reivindicación 9, CARACTERIZADA PORQUE los dos lipopéptidos se seleccionan entre el grupo que consiste de compuestos de tipo iturina, compuestos de tipo surfactina y compuestos de tipo fengicina.

11. La composición de la reivindicación 10, CARACTERIZADA PORQUE los dos lipopéptidos se seleccionan entre el grupo que consiste de iturinas, surfactinas y compuestos de tipo fengicina.

12. La composición de la reivindicación 1, CARACTERIZADA PORQUE el lipopéptido es parte de o un extracto de un producto de fermentación que contiene lipopéptidos.

13. La composición de la reivindicación 12, CARACTERIZADA PORQUE el producto de fermentación que contiene lipopéptidos proviene de una bacteria de la especie *Bacillus*.

14. La composición de la reivindicación 13, CARACTERIZADA PORQUE la bacteria de la especie *Bacillus* se selecciona entre el grupo que consiste de *Bacillus subtilis* o *Bacillus amyloliquefaciens*.

15. La composición de la reivindicación 14, CARACTERIZADA PORQUE el *Bacillus subtilis* se selecciona entre el grupo que consiste de *Bacillus subtilis* QST713 y sus variantes.

16. La composición de la reivindicación 1, CARACTERIZADA PORQUE la proporción peso en peso entre el fungicida poliénico y el lipopéptido es de 100:1 a 1:100.

17. La composición de la reivindicación 1, CARACTERIZADA PORQUE la proporción peso en peso entre el fungicida poliénico y el lipopéptido es de 10:1 a 1:10.

18. La composición de la reivindicación 1, CARACTERIZADA PORQUE la proporción peso en peso entre el fungicida poliénico y el lipopéptido es de 5:1 a 1:5.

19. La composición de la reivindicación 1, CARACTERIZADA PORQUE la proporción peso en peso entre el fungicida poliénico y un lipopéptido es de 2:1 a 1:2.

20. La composición de la reivindicación 1, CARACTERIZADA PORQUE la proporción peso en peso entre el fungicida poliénico y el lipopéptido es de 1:1.

21. Una composición CARACTERIZADA PORQUE comprende un fungicida poliénico seleccionado del grupo que consiste de natamicina, nistatina y derivados de los mismos y un producto de fermentación que contiene lipopéptidos, en donde el lipopéptido es seleccionado del grupo que consiste de compuestos del tipo iturina, compuestos del tipo surfactina y compuestos del tipo fengicina y la relación peso a peso del fungicida poliénico y el lipopéptido es de 500:1 a 1:500.

22. La composición de la reivindicación 21 CARACTERIZADA PORQUE el producto de fermentación que contiene lipopéptidos proviene de una bacteria de la especie *Bacillus*.

Bacillus lipopeptides: versatile weapons for plant disease biocontrol

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In the context of biocontrol of plant diseases, the three families of *Bacillus* lipopeptides – surfactins, iturins and fengycins were at first mostly studied for their antagonistic activity for a wide range of potential phytopathogens, including bacteria, fungi and oomycetes. Recent investigations have shed light on the fact that these lipopeptides can also influence the ecological fitness of the producing strain in terms of root colonization (and thereby persistence in the rhizosphere) and also have a key role in the beneficial interaction of *Bacillus* species with plants by stimulating host defence mechanisms. The different structural traits and physico-chemical properties of these effective surface- and membrane-active amphiphilic biomolecules explain their involvement in most of the mechanisms developed by bacteria for the biocontrol of different plant pathogens.

Bacillus – a source of bioactive molecules

One of the biggest ecological challenges facing microbiologists and plant pathologists in the near future is the development of environmentally friendly alternatives to the extensive use of chemical pesticides for combatting crop diseases (Box 1). The use of beneficial microorganisms (biopesticides) is considered one of the most promising methods for more rational and safe crop-management practices [1].

Members of the *Bacillus* genus are often considered microbial factories for the production of a vast array of biologically active molecules potentially inhibitory for phytopathogen growth, such as kanosamine or zwittermycin A from *B. cereus* [2]. Their spore-forming ability also makes these bacteria some of the best candidates for developing efficient biopesticide products from a technological point of view. *Bacillus* spores have a high level of resistance to the dryness necessary for formulation into stable products.

One of the most commonly used and well-studied organisms, the rhizobacterium *B. subtilis*, has an average of 4–5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen structurally diverse antimicrobial compounds [3]. Among these antimicrobial compounds, cyclic lipopeptides (LPs) of the surfactin, iturin and fengycin (or plipastatin) families have well-recognized potential uses in biotechnology and biopharmaceutical applications because of their surfactant properties. Different groups of LPs can confer an advantage to the producing *Bacillus* strains in specific

ecological niches [4]. Iturin production seems to be restricted to *B. subtilis* [5] and *B. amyloliquefaciens* [6]. Surfactin or closely related variants such as lichenysin have been isolated from *B. coagulans* [7], *B. pumilus* and *B. licheniformis* [8], and fengycin production was identified in *B. cereus* [9] and *B. thuringiensis* [10] in addition to *B. subtilis* [11] and *B. amyloliquefaciens* [6]. This review highlights the importance of these LPs and the diversity of their modes of action in the global biocontrol activity of specific strains from *Bacillus* species. Recent advances show that they can act not only as ‘antagonists’ or ‘killers’ by inhibiting phytopathogen growth but also as ‘spreaders’ by facilitating root colonization and as ‘immuno-stimulators’ by reinforcing host resistance potential.

Different activities for structurally related compounds

Bacillus LPs are synthesized non-ribosomally via large multi-enzymes (non-ribosomal peptide synthetases, NRPSs; see Box 2) [3,12]. These biosynthetic systems lead to a remarkable heterogeneity among the LP products generated by *Bacillus* with regards to the type and sequence of amino acid residues, the nature of the peptide cyclization and the nature, length and branching of the fatty acid chain (Figure 1).

The surfactin family encompasses structural variants but all members are heptapeptides interlinked with a β -hydroxy fatty acid to form a cyclic lactone ring structure [8]. They are powerful biosurfactants with exceptional emulsifying and foaming properties. Because of their amphiphilic nature, surfactins can also readily associate and tightly anchor into lipid layers and can thus interfere with biological membrane integrity in a dose-dependent manner. Studies on lipid vesicles suggest that at low concentration [surfactin-to-lipid mole ratio (R_b) < 0.04 in the membrane] surfactins insert exclusively in the outer leaflet of the membrane inducing only limited perturbation. At intermediate concentration (R_b 0.05–0.1) they provoke a transient permeabilization, but membranes re-anneal. Irreversible pore formation then occurs at higher ratios (R_b 0.1–0.2) because of the insertion of surfactin-rich clusters in the membrane. Further addition of surfactins to reach the critical micellar concentration (CMC) leads to complete disruption and solubilization of the lipid bilayer with formation of mixed micelles (at R_b 0.22) [13,14]. Interestingly, the presence of cholesterol in the phospholipid layer attenuates the destabilizing effect of surfactins [14], which suggests that the susceptibility of biological

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Box 1. *Bacillus* sp. as a marketed biocontrol agent

The global market for phytosanitary products used worldwide to ensure crop yield was estimated at US\$26.7 billion in 2005 [73]. Synthetic pesticides dominate this market. However, irrational selection and use led to environmental toxicity of their residues, decrease or loss of efficacy due to adaptation of pathogens, or undesirable effects on non-target organisms sharing the ecosystem. Furthermore, homologation procedures for new chemical products request more and more supplementary toxicological, and thus expensive, studies and the consumer concern for safer food and for more ecologically compatible plant disease management practices is growing fast. The use of chemicals has therefore been declining since 2000 and will probably follow the same trend in the next few years. In parallel, new strategies have emerged, such as the development of biopesticides that can be broadly defined as living organisms, or natural products derived from these organisms, that are used to suppress pest populations. These organisms include plants (genetically modified crops), insects, nematodes and microorganisms. Their overall contribution to plant health management is currently relatively small, representing ~2.5% of total agricultural sales. However, the growth rate of biopesticides is estimated to be ~10% per annum for the next five years and is expected to reach a billion dollars by 2010. Among these biopesticides, microorganism-based products (bacteria, fungi, virus, yeasts) represent ~30% of total sales and new products are regularly brought to the market (see [73] for recent data about biopesticide market trends). These beneficial microbes, also termed

biological control (or biocontrol) agents, represent an environmentally friendly alternative to chemicals and offer different modes of action for combatting pathogens. Biocontrol can thus be used in situations where no control is currently available, where conventional pesticides cannot be used owing to residue concerns, or in the rapidly growing sector of organic farming.

These biocontrol agents can also be applied together with chemicals, either in rotation to reduce the possible development of pathogen resistance or in an integrated pest management strategy with the goal of minimizing the use of synthetic insecticides. Microbial products are used in field crops and greenhouses to reduce diseases on various cereals, legumes, fruits, flowers and ornamentals caused either by soil-borne, foliar or post-harvest pathogens. Most of the bacterial strains exploited as biopesticides belong to the genera *Agrobacterium*, *Bacillus* and *Pseudomonas* [1] (Figure 1). *Bacillus thuringiensis*, specifically devoted to insect pest control, accounts for >70% of total sales. As for the rest, *Bacillus*-based products represent about half of the commercially available bacterial biocontrol agents and these are *Bacillus subtilis* strain GBO3 (Gustafson company, USA, www.bayercropscience.com/gustafson), *B. subtilis* strain QST 713 (Agraquest, USA, www.agraquest.com), *B. subtilis* MBI 600 (Becker Underwood, USA, www.beckerunderwood.com), *B. subtilis* var. *amyloliquefaciens* strain FZB24 (Abitep GmbH, Germany, www.abitep.de), *B. licheniformis* strain SB3086 (Novozymes Biologicals, USA, www.novozymes.com) and *B. pumilus* strain GB 34 (Gustafson, USA).

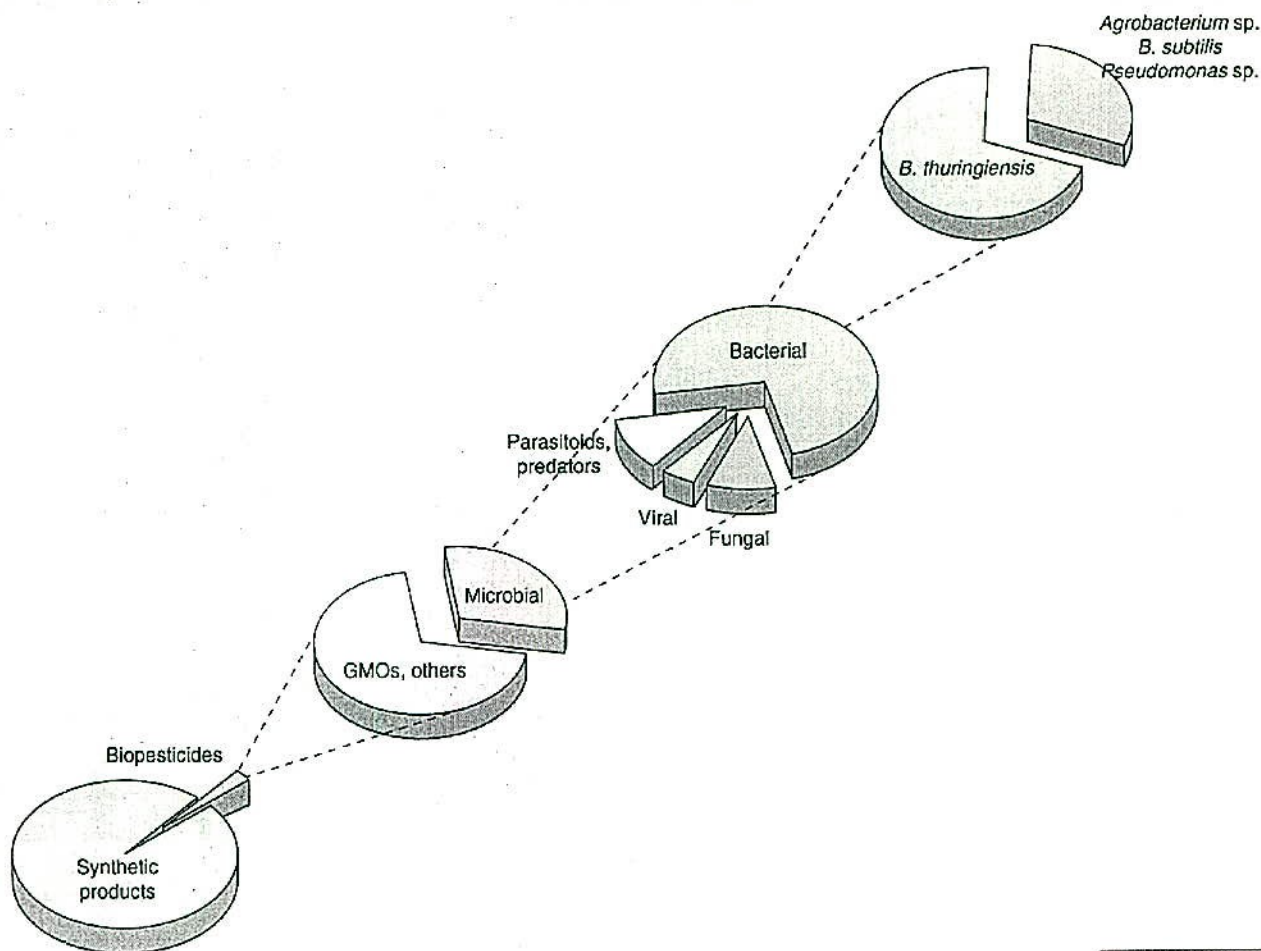


Figure 1. Market share for biological control agents [1,73].

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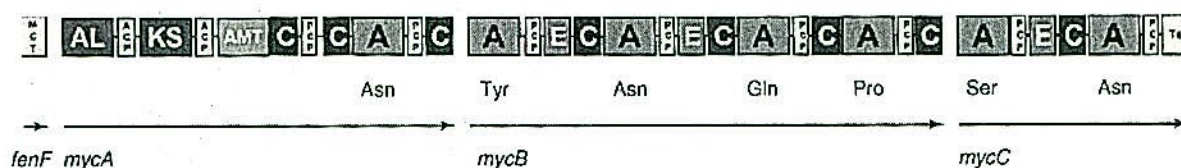
Box 2. Biosynthesis of lipopeptides in *Bacillus*

Lipopeptides from *Bacillus subtilis* are synthesized by non ribosomal peptide synthetases (NRPSs) or hybrid polyketide synthases and non ribosomal peptide synthetases (PKSs/NRPSs) (Figure 1). These modular proteins are responsible for the biosynthesis of several hundred bioactive compounds [74]. They are megaenzymes organized in iterative functional units called modules that catalyze the different reactions leading to polyketide or peptide transformation. Each module is subdivided into several catalytic domains responsible for each biochemical reaction. A typical NRPS module usually comprises ~1000 amino acid residues and is responsible for one reaction cycle of selective substrate recognition and activation as an adenylate (A-domain), tethering of a covalent intermediate as an enzyme-bound thioester (Peptidyl-Carrier-Protein, or PCP-domain), and peptide bond formation (Condensation, or C-domain) [12]. The basic set of domains within a module can be extended by substrate-modifying domains, including domains for substrate epimerization (E-domain), hydroxylation, methylation and heterocyclic ring formation, which are either inserted at specific locations into the module or act *in trans* as independent catalytic units. A thioesterase domain (Te-domain) is usually present in the last module to ensure the cleavage of the thioester bond between the nascent peptide and the last PCP-domain. In several cases, this thioesterase is responsible for the cyclization of the peptide.

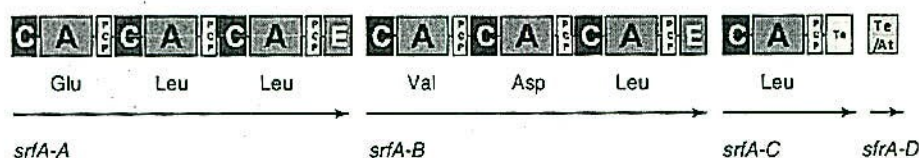
Three large open reading frames (ORFs) coding for surfactin synthetases are designated *srfA-A*, *srfA-B* and *srfA-C* [8]. They present a linear array of seven modules (one module per residue). Three modules are present in the products of *srfA-A* and *srfA-B* and the last

one in *srfA-C*. The fatty acid chain is added to the amino acid activated in the first module. A first thioesterase fused with the C-terminal end of the last activation PCP domain is responsible for the release of the synthesized product from the enzymatic template. A second thioesterase/acyltransferase (Te/At-domain) encoded by a fourth gene, *srfA-D* stimulates the initiation of the biosynthesis [75]. Similarly, plipastatin or fengycin are synthesized by NRPSs encoded by an operon with five open reading frames *ppsA-E* (or *fenA-E*) [76]. The first three enzymes contain two modules, the fourth contains three modules and the last enzyme consists of one module. Unlike surfactin and fengycin, iturin derivatives are synthesized by a PKS-NRPS hybrid complex [3,6,77]. The operon consists of four open reading frames called *fenF*, *mycA*, *mycB* and *mycC* or *ituD*, *ituA*, *ituB* and *ituC* for mycosubtilin or iturin, respectively. The last three genes code for the NRPSs responsible for the incorporation of the first residue for *mycA* (or *ituA*), the following four residues for *mycB* (or *ituB*) and the two last residues for *mycC* (or *ituC*). The difference between structures of iturin A and mycosubtilin (in which the last amino acids are inverted) can be explained by an intragenic domain change in *mycC* and *ituC*. *FenF* (*ituD*) encodes a malonyl-CoA transacylase (MCT-domain) and the *mycA* also contains genes related to polyketide syntheses. These genes are responsible for the last steps of the biosynthesis of the fatty acid chain (last elongation and β -amination) before its transfer to the first amino acid of the peptidic moiety (acyl-CoA ligase (AL-domain)), acyl carrier protein (ACP-domain), β -keto acyl synthetase (KS-domain), amino transferase (AMT domain)) [78].

Mycosubtilin operon



Surfactin operon



Plipastatin operon

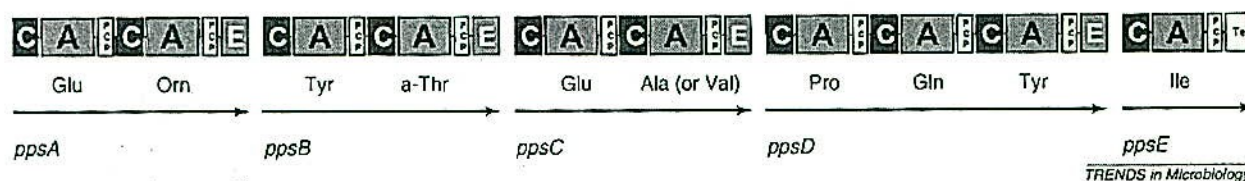


Figure 1. Operons of mycosubtilin, surfactin and plipastatin synthetases. Schematic representation of operons (ORFs, domains of NRPSs or PKSs and amino acid incorporated by the different modules) encoding catalytic machinery responsible for the biosynthesis of representative members of each family of lipopeptides produced by *Bacillus subtilis*: mycosubtilin for the iturin family, plipastatin for the fengycin family and surfactin. Domains are described in the box text.

membranes might vary in a specific manner depending on the sterol content of the target organisms. This could explain why surfactins display haemolytic, antiviral, antimycoplasmal and antibacterial activities but intriguingly, no marked fungitoxicity. This lipid bilayer destabilization process is facilitated by the three-dimensional form of the

surfactin molecule featuring charged side chains protruding into the aqueous phase and apolar moieties reaching into the hydrophobic core of the membrane [15,16]. The use of chemically modified or biosynthetic variants has revealed prominent roles for some sub-structures [8,17] (Figure 1).

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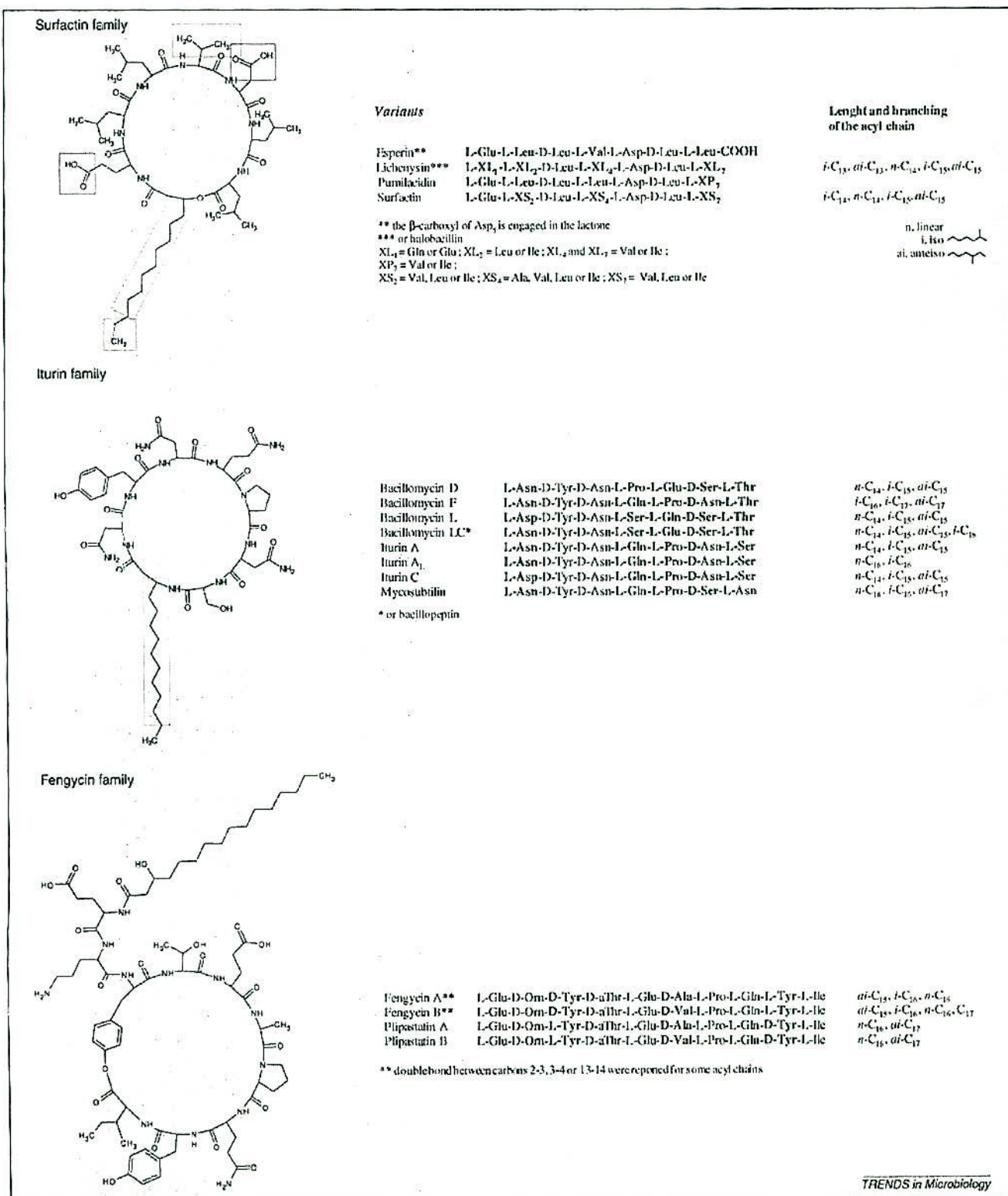


Figure 1. Structures of representative members and diversity within the three lipopeptide families synthesized by *Bacillus* species. Boxed structural groups are those that were shown to be particularly involved in interaction with membranes and/or are supposed to be important for biological activity in addition to the cyclic nature of the molecule [5,8,17]. To the best of our knowledge, no clear data are available to date for fengycins in this context. Boxed blue, type of branching (linear, iso, anteiso); boxed orange, acyl chain length; boxed red, ionisable or polar groups; boxed green, hydrophobicity of residue in position 4; boxed yellow, L-Asx(1)-D-Tyr(2)-D-Asn(3) sequence.

Iturin A and C, bacillomycin D, F, L and LC and mycosubtilin were described as the seven main variants within the iturin family (Figure 1). They are heptapeptides linked to a β -amino fatty acid chain with a length of 14 to

17 carbons. Though they are also strongly haemolytic, the biological activity of iturins is different to surfactins: they display a strong *in vitro* antifungal action against a wide variety of yeast and fungi but only limited antibacterial

and no antiviral activities [18–21]. This fungitoxicity of iturins almost certainly relies on their membrane permeabilization properties (see Figure 1 for structural features that influence this interaction [5]). However, the underlying mechanism is based on osmotic perturbation owing to the formation of ion-conducting pores and not membrane disruption or solubilization as caused by surfactins [22].

The third family of LPs comprises fengycins A and B, which are also called plipastatins. These molecules are lipodecapeptides with an internal lactone ring in the peptidic moiety and with a β -hydroxy fatty acid chain (C_{14} to C_{18}) that can be saturated or unsaturated (Figure 1). Fengycins are less haemolytic than iturins and surfactins but retain a strong fungitoxic activity, specifically against filamentous fungi [6,23,24]. Mechanistically, the action of fengycins is less well known compared with other LPs but they also readily interact with lipid layers and to some extent retain the potential to alter cell membrane structure (packing) and permeability in a dose-dependent way [25]. Lipopeptides are known to act in a synergistic manner as suggested by several studies on surfactin and iturin [26], surfactin and fengycin [27] and iturin and fengycin [6,28].

Implication of LPs in plant tissue colonization

Plants create a nutritionally and physico-chemically specific environment for microbes developing in the rhizosphere by constantly releasing a wide variety of low molecular weight compounds and macromolecules from their roots [29]. Some of these exuded molecules act as chemical signals for motile bacteria such as *Bacillus* sp. to move to the root surface, a process defined as chemotaxis. In the rhizosphere, biocontrol strains can use these root exudation products for persistence and growth [30]. This colonization process is a complex mechanism in which LPs can be involved at different levels.

Attachment and aggregation in microcolonies is the basis of plant root colonization by rhizobacteria [31]. Such microcolonies can be considered a type of biofilm [32] and it is thus generally assumed that microbial populations evolve and behave as biofilm communities on root and soil particle surfaces. In this context, studies have demonstrated the involvement of surfactins but not fengycins or iturins in pellicle formation at the air–water interface [24,33,34]. Spatial and temporal organization of cellular differentiation during the formation of biofilms was observed with several *Bacillus subtilis* isolates [33]. Interestingly, in contrast to strain ATCC21332, which rapidly died into the pellicle, strain ATCC9943 formed microcolonies allowing a continuous production of lipopeptides (P.J. *et al.*, unpublished).

More conclusively for the biocontrol field, Bais and collaborators have recently provided the first evidence for a role of surfactins in the formation of a stable biofilm by *B. subtilis* strain 6051 on *Arabidopsis* roots [35]. Deleting surfactin expression in this *Bacillus* strain led to the inability to form robust biofilms and reduced colonization rate and biocontrol of the disease caused by *Pseudomonas syringae*. Both surfactin and iturin modify the surface properties of *Bacillus subtilis* strains [36]. The specific activity of surfactin in cell adhesion and/or biofilm formation could be explained by its 3D topology, which exacerbates

its amphiphilic nature compared with the other families of LPs [5,8].

Bacterial colonies move over the root system to reach new niches with more nutrients. This can be achieved through the swarming process. This flagella-driven movement, which allows the bacteria to spread as a biofilm over the root surface, largely depends on the production of biosurfactants [37,38]. More specifically, several studies have shown that *Bacillus* LPs are crucially involved in such surface motility. Kinsinger and collaborators [34] have shown that surfactin and the potassium ion (which stimulates surfactin secretion) are important for the motility of *B. subtilis* strain 6051. It was recently demonstrated that surfactin, but also mycosubtilin, facilitate cell spreading of another *B. subtilis* isolate. These LPs function as wetting agents by reducing the surface tension [39].

Role of LPs in direct antagonism of phytopathogens

Once established in the phytosphere, *Bacillus* isolates can deploy their antibiotic arsenal. Reports suggesting a role for LPs in antagonism based only on *in vitro* antimicrobial activity are numerous. Here, we mainly consider selected studies that combine at least two other approaches in addition to intrinsic *in vitro* LP production to associate biocontrol activity and LPs. These approaches include the detection and quantification of the antibiotic compound in the microenvironment, previously inoculated with the producer strain, and the use of non-producing or overproducing derivatives and correlation with respective loss or increase in the biocontrol activity.

Surfactins have anti-bacterial properties *in vitro* and could be involved in biocontrol functions in the rhizosphere. Bais and collaborators provided evidence for this protective role of surfactin [35]. They showed that surfactin production is not only necessary for root colonization (as mentioned previously) but also for reduction of the infection caused by *Pseudomonas syringae* on *Arabidopsis* plants. This disease control was associated with the production of inhibitory quantities of surfactin at the root level. However, it remains unclear whether the protective effect is due to direct bactericidal activity or if it also indirectly depends on the inhibition of adherence of pathogenic cells to the root surface and therefore inhibition of biofilm growth. Surfactin also displays anti-viral activity through disintegration of the envelope, but to our knowledge no example of effective surfactin-related biocontrol of virus-mediated plant disease has been reported.

The involvement of iturins and fengycins was shown in the antibiosis-based biocontrol activity of *Bacillus* strains against various pathogens and in different plant species. In the case of soil-borne diseases, iturin A produced by *B. subtilis* RB14 is involved in damping-off of tomato (a seedling disease) caused by *Rhizoctonia solani* [40]. Overexpression of the closely related mycosubtilin in *B. subtilis* ATCC 6633 also led to a significant reduction of seedling infection by *Pythium aphanidermatum* [41]. In control of phyllosphere diseases, a contribution of both iturins and fengycins was recently shown in the antagonism of *B. subtilis* towards *Podosphaera fusca* infecting melon leaves [28]. This was demonstrated by identifying iturins and fengycins as the main antibiotic products excreted by

Box 3. Systemic resistance induced in plants by non-pathogenic microorganisms

Plants have evolved several inducible defence mechanisms against pathogen attacks. A first infection by an incompatible necrotizing pathogen can trigger a systemic resistance reaction that renders the host less susceptible to subsequent infection in distal tissues. This long-lasting phenomenon was termed systemic acquired resistance (SAR) [79]. Some non-pathogenic rhizobacteria can also reduce disease through the stimulation of inducible plant defence mechanisms that renders the host more resistant to further pathogen ingress, a phenomenon termed induced systemic resistance (ISR) [80,81]. Conclusive evidence for the role of ISR in disease reduction by a given bacterium tested on a particular pathosystem is obtained by verifying the spatial separation of the pathogen and the resistance-inducing agent to exclude any direct antagonistic interaction. ISR can be globally viewed as a three-step process, schematized in Figure 1. The list of bacteria identified as ISR inducers has grown rapidly over the past 15 years and includes Gram-negative bacteria and members of the *Pseudomonas* and *Serratia* genera, but also Gram-positive bacteria and more particularly, *Bacilli* [66,82] (Table 1). Most of these bacteria live freely in the rhizosphere soil but some inducers, such as *Rhizobium elti*, behave as endophytes.

Rhizobacteria-mediated ISR can occur in many dicotyledonous and monocotyledonous plant species. Analogous to pathogen-induced SAR, protection afforded through ISR is non-specific regarding the nature of the infectious agent. Because of its systemicity, the enhanced defensive capacity is expressed in roots as well as in leaves. ISR is thus effective against root-infecting pathogens, but can also reduce damage from pathogens that are active exclusively on foliage, flowers or fruits. Control of diseases caused by fungi, bacteria and viruses has been shown but ISR has also been a successful strategy in the management of nematode and insect pests in several crops. Of prime interest for fundamental comprehension of the phenomenon and for in-field applications as biopesticide products, it also appears that a particular bacterial strain can trigger resistance in the same plant against various pathogens. Multiple defence mechanisms can thus be stimulated in the host to respond adequately after invasion. Rhizobacteria-mediated ISR does not confer total protection against pathogen infection but ISR-based biocontrol strategies are promising because the phenomenon is long-lasting and not conducive to the development of pathogen resistance (due to multiplicity and variety of induced defence strategies) and some trials have been performed successfully under field conditions.

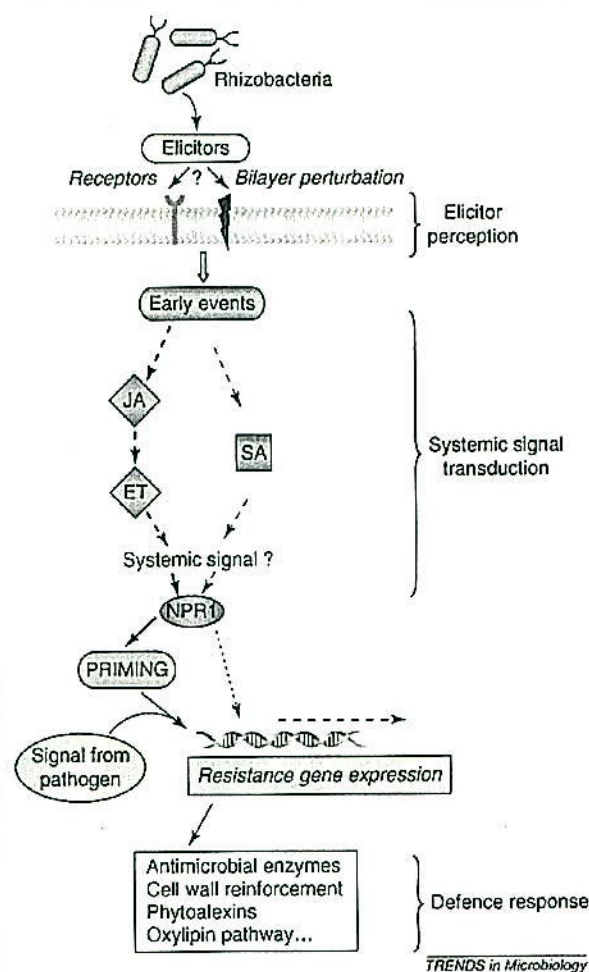


Figure 1. General scheme of ISR illustrating the three main steps of the phenomenon leading to enhanced responsive capacity of the host plant. The signal transduction pathway is in most cases controlled by the sequential sensitization of jasmonic acid (JA) and ethylene (ET) at the site of application of the inducing agent. As in the case of the systemic resistance induced by incompatible pathogens (SAR), rhizobacteria-mediated ISR required the positive regulator protein NPR1 that is activated following unknown systemic signaling events downstream to ET perception. This results in a priming effect meaning that the onset of the phenomenon is not usually accompanied by substantial transcriptional reprogramming in the host plant before the pathogen attack. It is likely that no direct transcriptional stimulation of the defence-related genes occurs upon non-pathogenic rhizobacteria recognition but rather the host resistance capacity is enhanced once the infection process is started. Alternatively, some non-pathogenic rhizobacteria were reported to trigger a salicylic acid (SA)-dependent signalling pathway that leads to a state of induced resistance resembling pathogen-dependent SAR (see [80-82] for recent reviews).

the strains, by showing the strong inhibitory effect of these LPs on *P. fusca* conidia germination, and by recovering LPs from bacterial-treated leaves and using LP-deficient transformants. *Bacillus subtilis* S499 efficiently produces LPs from the three families, and notably produces a wide variety of fengycins [11,42]. Direct evidence for a role of fengycins in disease reduction derives from experiments showing the ability of strain S499 to protect wounded apple fruits against gray mold disease caused by *Botrytis cinerea*. This role was demonstrated by the effective disease control provided by treatment of fruits with lipopeptide-enriched

extracts and by *in situ* detection of fengycins in inhibitory amounts [42].

With such a vast array of biological activities for LPs, *Bacillus* strains that efficiently produce the three families cannot act only on phytopathogens, and also influence other microbial populations enabling these bacteria to cope with competing organisms within the plant rhizosphere.

LPs as inducers of plant resistance

Fengycin- and surfactin-type LPs can interact with plant cells as a bacterial determinant for turning on an immune

Table 1. Tentative listing of *Bacillus* strains reported as plant systemic resistance inducers

Species, strain	Pathosystem
<i>B. subtilis</i> sp.	Cotton/ <i>Meloidogyne</i>
GB03	Cucumber/ <i>Erwinia</i> , beetle; <i>Arabidopsis</i> / <i>Erwinia</i>
IN937b	Tomato/Cucumber mosaic cucumovirus (CMV), Tomato mottle virus (TMV)
S499	Cucumber/ <i>Colletotrichum</i> ; Tomato/ <i>Pythium</i> ; Bean/ <i>Botrytis</i>
FZB-G	Tomato/ <i>Fusarium</i>
BacB	Sugar beet/ <i>Cercospora</i>
<i>B. pumilus</i> SE34	Tobacco/ <i>Peronospora</i> ; <i>Arabidopsis</i> / <i>Pseudomonas</i> ; Cucumber/beetle; Tomato/ <i>Fusarium</i> , <i>Phytophthora</i> , CMV, TMV
T4	Tobacco/ <i>Pseudomonas</i> ; <i>Arabidopsis</i> / <i>Pseudomonas</i>
INR-7	Loblolly pine/ <i>Cronartium</i> ; Cucumber/ beetle
203-6	Sugar beet/ <i>Cercospora</i>
<i>B. amyloliquefaciens</i> IN937	Tomato/CMV, TMV; Cucumber/beetle; <i>Arabidopsis</i> / <i>Erwinia</i>
EXTN-1	Tobacco/Pepper mild mottle virus (PMV); Cucumber/ <i>Colletotrichum</i> ; <i>Arabidopsis</i> / PMV
<i>B. thuringiensis</i> Berliner	Coffee/ <i>Hemileia</i>
<i>B. mycoides</i> BacJ	Sugar beet/ <i>Cercospora</i>
<i>B. pasteurii</i> C-9	Tobacco/ <i>Peronospora</i>
<i>B. sphaericus</i> B43	Potato/nematode
<i>B. cereus</i> B1	White clover/nematode

response through the stimulation of the induced systemic resistance phenomenon (ISR, Box 3). In bean and tomato, a role for surfactins and fengycins in plant defence induction was demonstrated by the similar protective activity of purified compounds compared with the activity directly from the producing strain [27]. More conclusively, a significant protective effect was gained by treating plants with various LP-overproducing derivatives generated from the wild-type *B. subtilis* 168, which is not able to synthesize these compounds and is not active on plants. The macroscopic disease reduction was related to metabolic changes associated with plant defence responses [27]. In tomato, LP-overproducing *Bacillus* isolates stimulate the two key enzyme activities of the oxylipin pathway, a metabolic route initiated by the lipoxygenase enzyme (LOX) [43]. Treatment of potato tuber cells with purified fengycins, but not surfactins and iturins, resulted in the accumulation of plant phenolics involved in, or derived from, phenylpropanoid metabolism [42]. This pathway is also well known to be stimulated concomitantly with the activation of plant defence reactions [44]. Major changes in defence-associated early events and in the phenolic pattern of tobacco cells on treatment with surfactins and fengycins were also observed (M.O. *et al.*, unpublished). Modification of this phenylpropanoid signature was concomitant with a significant accumulation of mRNAs coding for phenylalanine ammonia lyase (PAL), the entry enzyme of the metabolic route (M.O. *et al.*, unpublished). *B. subtilis* S499 can also stimulate a systemic defence response in cucumber leading to protection against *C. lagenarium* [45]. However, semi-purified LP extracts enriched in fengycins, surfactins and iturins failed to confer protection, suggesting that they are not involved in the resistance triggering process in that plant. The potential of LPs as plant resistance inducers is also supported by the recent work of Tran and collaborators on massetolide A [46]. This cyclic LP produced by *Pseudomonas fluorescens* retains ISR-eliciting activity in tomato plants for the control of *Phytophthora infestans* as demonstrated by testing the purified compounds and mutants impaired in their production.

As stated above, most of the biological activity of LPs is probably related to their effect on the lipid part of biological

membranes. However, the presence of LPs was not associated with any phytotoxicity or adverse effect on the integrity and growth potential of plant cells. This suggests that these molecules could interact without creating irreversible pore formation but in a way capable of inducing disturbance or transient channelling in the plasma membrane that can in turn activate a biochemical cascade of molecular events leading to defensive responses. Interestingly, a similar hypothesis has recently been put forward to explain the anti-proliferative effect of surfactin on colon cancer LoVo cells that did not appear to be leaky or lysed but underwent significant changes in the expression of protein factors regulating cell survival [47].

We are far from completing a comprehensive picture of the molecular events underlying lipopeptide-induced ISR. However, in light of these results, it seems that each family retains a specific ability to stimulate different plant cells. Surfactins interact with bean, tomato and tobacco but not potato, whereas fengycins can elicit a response in the three solanaceae species tested to date. None of the LPs tested so far have an effect on cucumber. Intriguingly, ISR-inducing activity of iturins is not observed on treatment of tomato plants, potato tuber slices or tobacco cells. The presence of ergosterol in fungi and yeast plasma membranes is important for iturin activity against these microorganisms [5]. The lack of effect on plant cells could be because of a different composition in phytosterols mainly composed of sitosterol, stigmasterol, campesterol and in some species cholesterol, leading to an attenuated membrane-disruptive property.

Ecological fitness of *Bacillus* in LP production

Clues from *in vitro* studies

The study of *Bacillus* fitness in the rhizosphere in terms of antibiotic synthesis and induction of systemic resistance is crucial for optimizing biocontrol strategies. This is a challenging issue given the specificity of the nutritional context imposed by the host plant, the complexity of microbial communities sharing the ecosystem and the variability of physico-chemical parameters inherent to the soil, such as mineral content, pH, temperature and oxygen availability. Availability of carbon and nitrogen sources can

drastically influence the production of biocontrol determinants by rhizobacteria as demonstrated with various pseudomonads [48,49]. *In vitro* experiments have also provided some insights into the effect of different substrates (e.g. sugars, nitrogen source and iron) and physicochemical conditions (e.g. oxygen availability, temperature and pH) on LP synthesis by *Bacillus* strains [8,11,50,51].

From a physiological point of view, rhizosphere conditions can also modulate antibiotic gene expression. Bacterial population development is restricted by nutrient availability owing to limited root exudation rate [29,31,52]. Microbial cells on roots can thus be in a nutrient-starved state that would impose specific growth conditions and affect growth rates. In artificial media, cells in the transition from exponential phase to stationary phase mostly produce surfactins – fengycin synthesis is delayed to early stationary phase, and iturins only accumulate later [6,53]. This implies that rhizosphere conditions can drive cell physiology to a given growth rate that in turn could favour the production of one specific LP family. Also, transcriptional mechanisms such as quorum sensing are related to biofilm formation [54] and thus have a crucial role in the regulation of LPs *in situ* as has been shown for other biocontrol secondary metabolites [55]. Surfactin production is quorum sensing-dependent and involves two pheromones, ComX and PhrC, together with several transcription factors such as CodY, DegU and PerR, which form a complex cascade governing multiple differentiation pathways such as sporulation and competence [56]. Duitman *et al.* [57] have shown recently that mycosubtilin synthetase expression is under the influence of another regulatory cascade depending on AbrB, one of the main transition state regulators in *B. subtilis*. In contrast to surfactin, the expression of mycosubtilin synthetase is lowest in minimal medium and highest in rich medium. Cosby and collaborators [51] also showed that surfactin synthetase expression is pH-dependent and we recently demonstrated that mycosubtilin synthetase expression is oxygen-dependent [50]. To our knowledge, little information is available about the regulation mechanism of fengycin biosynthesis.

LP production and gene expression *in situ*

As described in the previous section, LPs from all three families were occasionally detected in the phytosphere, illustrating that natural conditions are conducive for their biosynthesis, at least in some cases. LP production was demonstrated for *Bacillus* populations growing on roots, leaves and fruits [27,28,35,40,58] but such examples are rare. Quantities produced in the rhizosphere are difficult to estimate because of the small amounts excreted relative to the other organic compounds present in the environment, the difficulty in extracting LPs from the complex soil matrix, and the possibility that low quantities produced are restricted from diffusing freely and can be rapidly embedded in the membrane structure of the target organism. Analytical methods such as matrix-assisted laser desorption ionization-time of flight mass (MALDI-TOF) spectrometry and electrospray ionization mass spectrometry (either coupled with liquid chromatography or not coupled) were effective for LP measurement in various extracts and in whole cells [59,60]. Scanning mass spec-

trometry could also be a promising technique for such purposes. These analytical tools should be more thoroughly exploited for reliable qualitative and quantitative measurement of LPs produced in the rhizosphere, together with the development of optimized methods for the recovery of LPs from the soil. Immunological detection by competitive ELISA assays can provide a sensitive alternative to these analytical methods for direct quantification and localization of LPs on the surface of plant tissues without prior extraction with organic solvents [61].

However, some abiotic and nutritional culture conditions influence the pattern of LPs produced by *B. subtilis* strains. Peptidic and/or lipidic variants with altered or improved biological properties could therefore be naturally synthesized by *Bacillus* depending on the nutritional context and other factors [5,8,62]. Rhizosphere-specific parameters might also influence the types and amounts of LPs synthesized in the soil.

Few studies, if any, have been devoted to investigating LP gene expression during interaction with plants, despite the fact that a variety of genetic approaches are available for the analysis of global gene expression patterns *in situ*. Examples of these technologies include promoter-trapping technologies and microarray-based transcriptomic and functional genomic techniques that offer high-throughput alternatives for the identification of genetic traits specifically expressed *in planta*. These techniques were successfully applied for detection of genes from beneficial enterobacteria, pseudomonads and Rhizobia activated in the phytosphere [63,64]. Reporter gene technology (fusion of the promoters of the genes of interest to reporter genes such as *lacZ*, *xylE*, *gusA*, *luxAB* or *gfp*) is probably the most powerful approach for analysis of *Bacillus* rhizosphere competence and LP gene expression *in situ* as has been shown with *Pseudomonas* [65]. The overall lack of information on LP gene expression during plant interaction is in part due to the fact that *Bacillus* has been studied primarily for its biochemical and genetic traits, and research on this species as a biocontrol agent has focussed mainly on the efficacy of the protective effect under practical use in the field or greenhouse [66]. In addition, the natural competence of *B. subtilis* for taking exogenous DNA is well recognized in strains grown in the laboratory, but most of the valuable environmental strains are highly recalcitrant to transformation [27,57].

Concluding remarks and future perspectives

LPs are involved in most of the mechanisms described to date that explain the biocontrol effect of different strains (Figure 2). This broad range of activities is due to the physico-chemical properties of these amphiphilic molecules. Reduction of surface tension, modification of surface properties and perturbation of lipid bilayers are different traits characteristic of surfactant molecules. However, compared with many classical surfactants, these molecules have relatively low critical micellar concentration (CMC) [15]. In the case of surfactin, this is clearly correlated with the haemolytic activity on erythrocyte suspensions and CMC is thus an indicator of the minimum concentration at which there is some effect on eukaryotic cells. This rule cannot be extended to the iturin family

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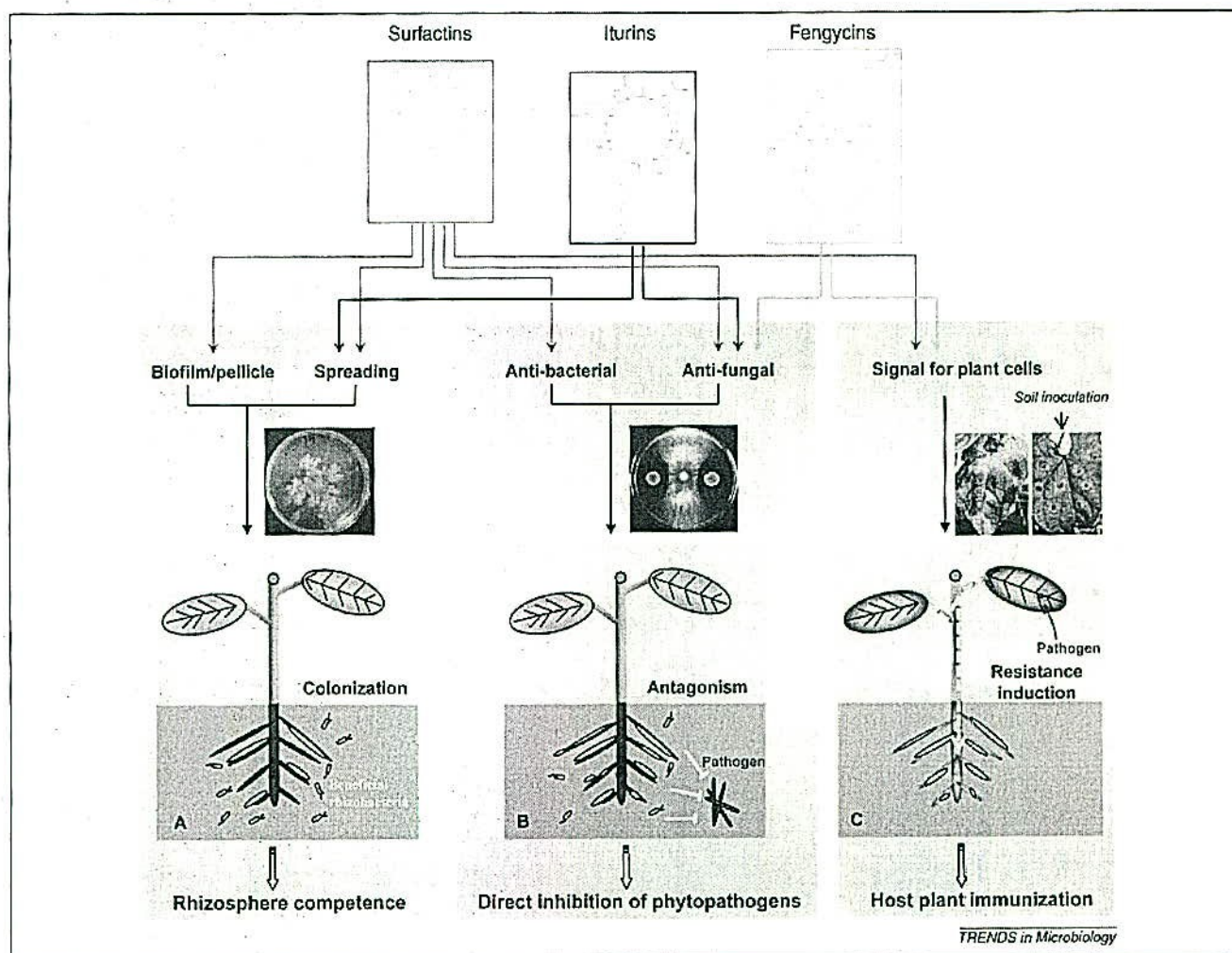


Figure 2. Overview of *Bacillus* lipopeptide interactions in the context of biological control of plant diseases. From left to right, the three photographs show bacterial spreading, fungal growth inhibition through the production of fungitoxic compounds by blue bacterial cells and leaf disease reduction following inoculation of the beneficial bacterium on roots. They illustrate how to get experimental indications about the potential involvement of one particular strain in the three phenomena schematically represented in (A), (B) and (C). Establishment in biofilm and/or microcolonies of the rhizobacterium is represented in (A), (B) represents direct antibiosis that can be exerted by the established biocontrol strain toward pathogens sharing the same microenvironment. In (C), the arrows illustrate the emission of a signal following perception of the rhizobacterium at the root level. This signal moves over the entire plant leading to some systemic reinforcement allowing pathogen restriction at distal sites of infections (see Box 3).

(P.J., unpublished) and it has been shown that this molecule aggregates differently [67]. At higher concentrations than CMC, these molecules tend to be organized in lamellar vesicles instead of simple micelles. A comparison between the activities of the different variants of the three families of lipopeptides produced by *Bacillus subtilis* shows that the amphiphilic character is not the sole trait explaining these biological activities. Specific functions present in the peptidic moiety are also important.

LPs constitute a novel class of microbial-associated molecular patterns that can be specifically perceived by plant cells as signals for activating defence mechanisms. However, there is no partial common peptide sequence shared by the three families of LPs considered here, nor is there with other peptide products retaining ISR-eliciting activity such as flagellins or pyoverdins [68,69] or, more broadly, with molecular patterns identified in the interaction of pathogenic microorganisms with plant cells [70]. It is therefore important to search for putative receptors and to appreciate the relative importance of specific LP

substructures for ISR elicitation to understand the molecular aspects of LP perception by plant cells (a list of similar outstanding questions is given in Box 4). Some advances have been made recently in our understanding of LP interaction with biological membranes but this has mainly relied on physico-chemical studies using artificial phospholipid bilayers. The broadest panoply of variants should thus be tested for their biological effect directly on

Box 4. Outstanding questions

- Can we relate the capacity of a particular strain to form microcolonies in biofilm and the potential for successful plant colonization and LP production?
- What is the level of *in situ* LP production by *B. subtilis* cells in soil and what are the rhizosphere factors that modulate LP synthesis?
- What is the molecular basis for LP-induced plant systemic resistance?
- Are there specific receptors to LPs involved in the interaction with eukaryotic cells?
- Can LP signature be used as screening tool for selecting efficient biocontrol strains?

living cells. Data should be evaluated with regards to the changes induced in the 3D conformation of these variants.

This review highlights the competitive advantage of efficient production of surfactin, fengycin and iturin with specific roles and targets. Within each family, some structural homologues are seemingly more active than others. The variety of the LPs naturally synthesized can thus explain why some *Bacillus* strains are more efficient in reducing plant diseases than others. Mass spectrometry methods developed to rapidly characterize the LP signature of particular isolates using whole cells (thus avoiding cultivation and extraction) could be exploited as a powerful and rapid method of screening for the selection of useful strains in the context of biocontrol.

Efficiency of biological control in field or greenhouse trials has been improved by combining microorganisms with different modes of action [71,72]. This implies that every component of the consortium must be produced separately before application and, after inoculation, each of them should efficiently colonize the environment without providing any negative effect on the development of the others. The selection of single strains evolving diverse mechanisms to reduce disease incidence is thus of prime interest. *Bacillus* isolates that co-produce the three LP families should display multi-faceted biocontrol activity.

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