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Programa de Doctorado en Biología Molecular y Celular

Aplicación de sistemas de control genético e interacción biológica para la activación de agrupaciones génicas silenciosas en *Streptomyces* spp.

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Aplicación de sistemas de control genético e interacción biológica para la activación de agrupaciones génicas silenciosas en *Streptomyces* spp.	Inglés: Application of genetical control and biological interaction systems for silent gene clusters activation in *Streptomyces* spp.

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RESUMEN (en español)

Los productos naturales y sus análogos estructurales tienen gran relevancia en diversos campos de la industria como la farmacéutica, alimentaria, y cosmética debido a su amplia variedad química y de propiedades biológicas asociadas. Una fuente importante de compuestos naturales son los metabolitos secundarios microbianos, que exhiben propiedades de interés como antibacterianos, antifúngicos, antitumorales, antioxidantes, sideróforos, etc. En este contexto, merece destacar los metabolitos producidos por hongos y por el grupo bacteriano de los Actinomicetos; y en concreto el género *Streptomyces*. Los estreptomicetos son responsables de la biosíntesis del 70-80% de los compuestos naturales con propiedades bioactivas de interés. El desarrollo de técnicas de secuenciación ha puesto en relieve el enorme potencial de este grupo para la biosíntesis de metabolitos secundarios estructuralmente diversos. Sin embargo, la mayor parte de los genes implicados en la síntesis de estos compuestos se encuentran silenciados en condiciones estándar de cultivo de laboratorio, y, por tanto, sus productos no son conocidos. Dada la creciente aparición de microorganismos resistentes a los fármacos de uso habitual, así como a la aparición de enfermedades emergentes, se ha acentuado la necesidad de búsqueda de nuevos compuestos bioactivos que puedan servir como alternativas terapéuticas. Es por esto que la comunidad científica ha centrado grandes esfuerzos en tratar de activar rutas metabólicas silenciadas y poder caracterizar así los productos de las mismas.

Existen diversas estrategias para tratar de activar estas vías silenciadas como puede ser la aplicación de aproximaciones OSMAC, ingeniería de promotores, expresión heteróloga, ingeniería ribosómica, uso de elicitores, etc. Una técnica eficiente es la expresión heteróloga de genes reguladores, globales o específicos de ruta, así como de genes de mantenimiento conservados o *housekeeping*. En este trabajo, se clonaron cinco reguladores globales y cinco genes *housekeeping*, conocidos por inducir la

activación o la sobreexpresión de metabolitos secundarios en *Streptomyces coelicolor*, en dos construcciones separadas y se expresaron en 12 cepas de *Streptomyces* sp. Estas cepas fueron aisladas de hormigas cortadoras de hojas de la tribu *Attini* de Perú, lo cual supone el empleo de cepas aisladas de ambientes poco explorados. Además del empleo de cepas silvestres, los ensayos de expresión heteróloga fueron realizados sobre cepas resistentes a estreptomicina y rifampicina obtenidas por ingeniería ribosómica. El cultivo en diferentes medios de las mismas, dio lugar a cambios en el perfil metabólico en comparación con las cepas silvestres, observándose inhibición, activación y sobreproducción de compuestos.

Otra estrategia de interés, es el cocultivo de diferentes cepas de *Streptomyces.* En este trabajo se han realizado cocultivos sin contacto físico en los que solo existe intercambio de compuestos volátiles entre las cepas. Los ensayos realizados mostraron como ciertas cepas aumentaban su producción, comenzaban a producir nuevos compuestos o mejoraban su capacidad de crecimiento en respuesta a los volátiles producidos en el cocultivo. Esto pone en relieve el importante papel que tienen determinados compuestos volátiles, ya que pueden actuar como agentes inductores o inhibidores del metabolismo y del crecimiento. Además, se evaluó el potencial antifúngico del volatiloma de las diferentes cepas de estreptomicetos frente a *Escovopsis weberi*, patógeno natural que amenaza la estabilidad de los nidos de las hormigas donde las bacterias fueron aisladas. Esto refleja, no sólo el gran potencial bioactivo de estos compuestos, sino que además resalta la importancia ecológica de los compuestos volátiles en la interacción entre microorganismos.

RESUMEN (en Inglés)

Natural products and their structural analogues have great relevance in several fields of industry such as pharmaceuticals, food, and cosmetics, due to their wide chemical variety and wide range of biological properties. An important source of natural compounds is microbial secondary metabolites, which exhibit interesting properties such as antibacterial, antifungal, antitumor, antioxidants, siderophores, etc. In this context, it is worth highlighting the metabolites produced by fungi and the bacterial group of Actinomycetes; and specifically, the genus *Streptomyces*. Streptomycetes are responsible for the biosynthesis of 70-80% of natural compounds with interesting bioactive properties. The development of sequencing techniques has underlined the enormous potential of this group for the biosynthesis of structurally diverse secondary metabolites. However, the majority of the genes involved in the synthesis of these are silenced under standard laboratory culture conditions, and, therefore, their products are not known. Owing to the growing appearance of microorganisms resistant to common drugs, as well as the appearance of emerging diseases, the need to search for new bioactive compounds that can serve as therapeutic alternatives has increased. Due to this, the scientific community has focused great efforts on activating silenced metabolic

pathways and thus being able to characterize their products.

There are various strategies to activate these silenced pathways, such as the application of OSMAC approaches, promoter engineering, heterologous expression, ribosomal engineering, use of elicitors, etc. An efficient technique is the heterologous expression of regulatory, global or pathway-specific genes, as well as housekeeping genes. In this work, five global regulators and five housekeeping genes, known to induce activation or overexpression of secondary metabolites in *Streptomyces coelicolor*, were cloned in two separate constructs and expressed in 12 strains of *Streptomyces* sp. These strains were isolated from leaf-cutting ants of the *Attini* tribe of Perú, which implies the use of strains isolated from little-explored environments. In addition to the use of wild-type strains, heterologous expression assays were performed on streptomycin- and rifampicin-resistant strains obtained by ribosomal engineering. Their culture in different media leads to changes in the metabolic profile in comparison to wild strains, observing inhibition, activation, and overproduction of compounds.

Another strategy of interest is the coculture of different *Streptomyces* strains. In this work, cocultures without physical contact have been performed with the only exchange of volatile compounds between the strains. The tests carried out showed how certain strains increased their production, started to produce new compounds or improved their growth capacity in response to the volatiles produced in the co-culture. This highlights the important role that certain volatile compounds have, since they can act as inducers or inhibitory agents of metabolism and growth. Furthermore, the antifungal potential of the volatilome of the different strains of streptomycetes was evaluated against *Escovopsis weberi*, the natural pathogen that threatens the stability of the ant nests from where the bacteria were isolated. This points at, not only the bioactive potential of these compounds but also focuses attention to the ecological importance of volatile compounds in the interactions between microorganisms.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOLOGÍA MOLECULAR Y CELULAR

En primer lugar, agradecer a Carlos Olano y a Mónica G. Malmierca haberme brindado esta oportunidad, por todos los consejos y el apoyo durante estos años. Esto es por, y para vosotros.

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"I was taught that the way of progress
is neither swift nor easy"

-Marie Curie

Índice

I. Introducción

1-Productos naturales

Los productos naturales, así como sus análogos estructurales de origen sintético, tienen gran relevancia en industria farmacéutica, alimentaria y cosmética debido a su gran variedad química y la amplia gama de propiedades biológicas asociadas (Atanasov *et al*., 2021). En este sentido, a pesar de la multitud de moléculas sintéticas existentes, estas nunca han alcanzado la diversidad, complejidad y riqueza que caracteriza a los productos naturales, por lo que su estudio y descubrimiento ha despertado gran interés desde hace décadas. En 1945 Alexander Fleming, Ernst Chain y Howard Florey obtuvieron el Premio Nobel de Medicina por el descubrimiento de la producción de penicilina por *Penicillium chrysogenum*; y unos años después, en 1952, se concedió el Nobel de Fisiología a Selman A. Waksman por el descubrimiento de la estreptomicina sintetizada por *Streptomyces griseus*. Estos acontecimientos supusieron un punto de inflexión que impulsó el origen de la conocida como "era dorada" del descubrimiento de compuestos naturales, comprendida entre los años 1950 y 1960, donde el aislamiento de nuevas moléculas era relativamente frecuente (Hutchings *et al*., 2019; Shen, 2015). El hallazgo de gran cantidad de metabolitos con propiedades bioactivas, consiguió revolucionar el campo de la industria, y en especial la farmacéutica, permitiendo así salvar millones de vidas. Sin embargo, con el transcurso del tiempo, los avances en el descubrimiento de nuevos compuestos fueron disminuyendo, llegando a ser escasas las moléculas que alcanzan a ser empleadas en clínica. Esto, además, está motivado por el largo proceso de aprobación de un fármaco de uso en humanos, ya que se precisan de unos 10 a 15 años, lo que además conlleva considerables costes económicos (da Cunha *et al*., 2019; Takahashi *et al*., 2018).

A pesar de estas dificultades, existe una clara necesidad de continuar con la búsqueda y estudio de nuevos compuestos naturales. Por un lado, la aparición de resistencias a los fármacos actuales cada vez es más frecuente, destacando las resistencias a antimicrobianos debidas al uso indiscriminado y negligente de antibióticos en medicina humana y veterinaria, así como su empleo en ganadería y agricultura (Kasimanickam *et al*., 2021; Manyi-Loh *et al*., 2018). Muchas bacterias patógenas son resistentes a los tratamientos de uso actual y se pueden transmitir fácilmente a través de la cadena alimentaria o diseminarse en el medio ambiente, lo que supone un importantísimo problema de salud pública causando gran número de muertes y enormes costos económicos en el sistema sanitario (Doron *et al*., 2008). En especial, destaca el grupo de

las bacterias ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa*, y *Enterobacter* sp.) que son responsables de una amplia gama de infecciones intrahospitalarias y progresivamente se están volviendo resistentes a los antibióticos de uso habitual lo que supone uno de los mayores retos de la práctica clínica (De Oliveira *et al*., 2020). La Organización Mundial de la Salud (OMS) estima que cada año se producen casi cinco millones de muertes debido a bacterias resistentes a los antimicrobianos y calcula que en el año 2050 se convertirá en la principal causa de mortalidad con diez millones de muertes anuales (de Kraker *et al*., 2016; Thompson, 2023). Este factor, junto con el incremento de enfermedades emergentes, culmina por reclamar una elevada necesidad de búsqueda de nuevos compuestos naturales que puedan servir como alternativas terapéuticas a los medicamentos de uso actual. Un desafío habitual al que se enfrenta este campo de investigación es el continuo redescubrimiento de compuestos previamente descritos, por lo que cada vez es más necesaria la implementación de nuevas técnicas y aproximaciones que permitan descubrir nuevos compuestos bioactivos (Atanasov *et al*., 2021; Cloeckaert *et al*., 2020).

Los metabolitos secundarios producidos por plantas son una potente fuente de compuestos con propiedades bioactivas y constituyen la base de más del 25% de los fármacos existentes. La mayoría de estos, se generan como respuestas químicas defensivas ante condiciones de estrés tanto bióticas como abióticas, destacando entre sus múltiples actividades biológicas las propiedades antioxidantes y antiinflamatorias (Yeshi *et al*., 2022). Asimismo, los compuestos producidos por microorganismos también son un formidable recurso natural de interés por su potencial como antimicrobianos, antivirales, antioxidantes, sideróforos, pigmentos, hormonas de crecimiento, vitaminas, antitumorales, etc. Entre todos los metabolitos microbianos conocidos destacan los producidos por hongos y por el grupo bacteriano de los actinomicetos, dadas las numerosas actividades biológicas asociadas a estas moléculas (Rani *et al*., 2021).

El desarrollo de las técnicas de secuenciación masiva, así como la creciente comprensión sobre la biosíntesis de este tipo de compuestos ha motivado la continuidad de esta línea de investigación centrada en el descubrimiento y estudio de metabolitos microbianos y sus propiedades, así como la generación de nuevos derivados (Rani *et al*., 2021; Takahashi *et al*., 2018).

2-Los actinomicetos

Los actinomicetos son un heterogéneo grupo de bacterias Gram positivas, de vida libre, encontradas en variedad de ambientes. Poseen un elevado contenido en Guanina + Citosina (G+C) en su ADN (entre el 57% y el 75%) (Nazari *et al*., 2022) y presentan gran variedad morfológica, con integrantes con forma de cocos como el género *Microcroccus* sp., con forma de pequeños filamentos ramificados como el género *Nocardia* sp., o hifas ramificadas altamente diferenciadas como en *Streptomyces* sp. (Selim *et al*., 2021).

La mayoría de sus integrantes son saprófitos, habitando el suelo, aunque también hay miembros que conviven en ambientes acuáticos, en el aire o en asociación con otros organismos vivos como hongos, insectos, esponjas o plantas (Takahashi *et al*., 2018; van der Meij *et al*., 2017). En el suelo se encuentran en densidades entre 10^6 y 10^9 células por gramo de suelo, con preferencia por los suelos con alta carga de materia orgánica. Además, presentan un papel fundamental en varios procesos como pueden ser la descomposición de polisacáridos complejos y materia celulósica, la fijación de nitrógeno o la solubilización de fosfatos, contribuyendo así al reciclaje de nutrientes (Javed *et al*., 2021; Takahashi *et al*., 2018). Son organismos mesófilos, con temperaturas óptimas de crecimiento entre 25 ºC y 30 ºC y con preferencias por ambientes con pH neutro (entre seis y nueve) y con baja humedad (Nazari *et al*., 2022).

Este grupo conforma el amplio filo Actinobacteria (Figura 1) siendo la mayoría de sus representantes aerobios, aunque existen cepas anaerobias como *Actinomyces israelii* causante de actinomicosis en humanos (Anandan *et al*., 2016; Valour *et al*., 2014). Este último género, junto a otros como *Nocardia* o *Mycobacterium* pertenecen al grupo de actinomicetos oportunistas, que pueden causar enfermedades en el ser humano (Siavashifar *et al*., 2021).

Los actinomicetos constituyen uno de los grupos bacterianos más grandes, destacando por ser importantes productores de metabolitos con diversidad de estructuras químicas, así como múltiples propiedades bioactivas (Barka *et al*., 2016; De Simeis *et al*., 2021). Se estima que es el responsable de la producción de más de la mitad de sustancias bioactivas de origen microbiano y productor de dos tercios de los antibióticos de uso actual. En este contexto, destaca el género *Streptomyces*, aunque es importante tener presente que una interesante fuente de producción también son aquellos conocidos como

"actinomicetos raros", aislados de ambientes más particulares y, por lo tanto, su metabolismo no está tan estudiado. A partir de ellos se han identificado compuestos con actividades de interés como pueden ser la fortimicina aislada de *Micromonospora olivaceus*, la eritromicina producida por *Saccharopolyspora erythraea* o la rifamicina sintetizada por *Amycolatopsis mediterranei*, siendo los tres potentes antimicrobianos (Takahashi *et al*., 2018).

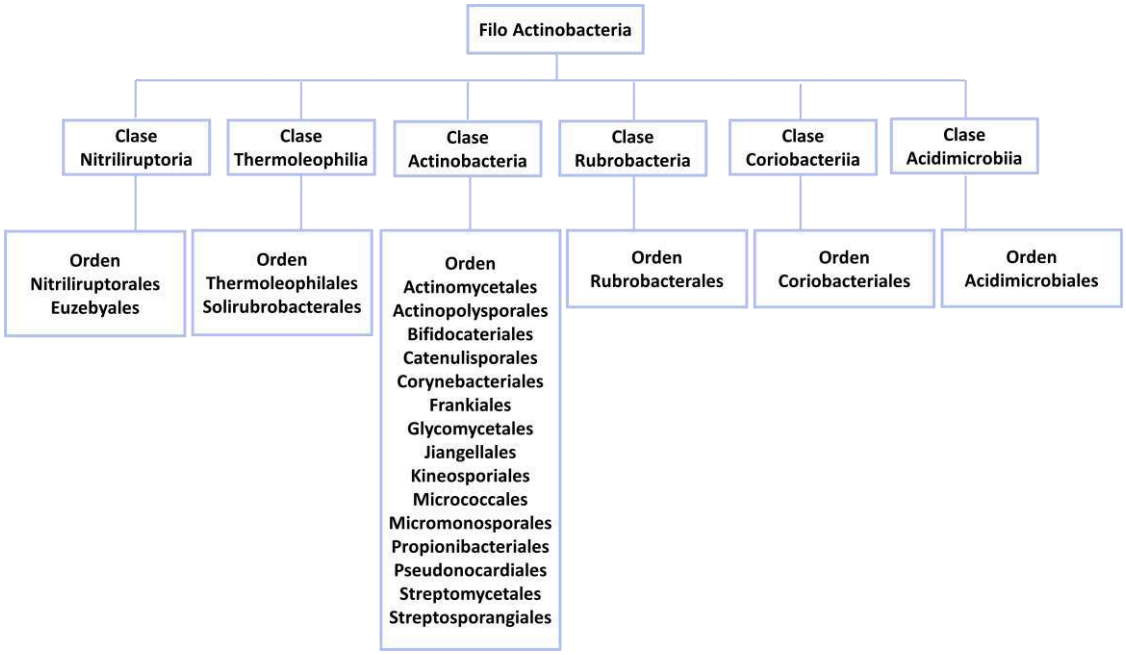


Figura 1: clasificación del filo Actinobacteria (Gao *et al*., 2012).

2.1-El género *Streptomyces*

El género *Streptomyces* es el miembro más representativo del grupo de los actinomicetos. En el suelo constituyen el 95% de los actinomicetos encontrados, aunque también colonizan ambientes marinos y otros ambientes poco explorados, incluso conviven asociados a otros seres vivos en relaciones endofíticas, de mutualismo o simbiosis (Alam *et al*., 2022). La mayor parte de los representantes de este grupo no son patógenos, con excepciones que afectan a humanos como *S. somaliensis* o *S. sudanensis*, o afectando a plantas como *S. scabies* (Kirby *et al*., 2012; Kotrbová *et al*., 2022).

Clasificado dentro de orden *Streptomycetales*, este género se caracteriza por presentar una morfología filamentosa y pared de peptidoglicano que le confiere capacidad de supervivencia en diversos ambientes. A pesar de que son bacterias inmóviles, su ciclo

de vida complejo consta de procesos de diferenciación celular, que conducen a la formación de esporas, estructuras de resistencia que permiten la diseminación (de Lima Procópio *et al*., 2012; Hasani *et al*., 2014).

Los estreptomicetos son responsables de la producción de aproximadamente el 70-80% de los compuestos naturales con propiedades bioactivas de diferente índole (Alam *et al*., 2022) y muchas de ellas con interés en industria farmacéutica como antitumoral (antraciclina, bleomicina) (Jose *et al*., 2021), antibacteriana (neomicina, estreptomicina), antifúngica (candicidina, nistatina) (Alam *et al*., 2022), antiviral (virantmicina, xiamicina D) (Jose *et al*., 2021; Kim *et al*., 2016), inmunosupresora (rapamicina, tacrolimus) (Alam *et al*., 2022; Jose *et al*., 2021; Kim *et al*., 2014), y antiparasitaria (ivermectinas, milbemicina D) (Shiomi *et al*., 2004). En agricultura, muchos compuestos producidos por estreptomicetos son promotores del crecimiento y desarrollo de plantas, por lo que son empleados como fertilizantes (Olanrewaju *et al*., 2019). Además, inducen resistencia a estreses en las mismas (Dow *et al*., 2023), ya que generan compuestos como sideróforos que se emplean como agentes de biocontrol; o trimetilamina, que induce cambios de pH en el ambiente, evitando la supervivencia de otros microorganismos que puedan afectar a cultivos. Este último, no solo alivia los estreses bióticos, sino que además supone una mayor disponibilidad de nutrientes en el suelo, así como favorece la solubilización de fosfatos (Olanrewaju *et al*., 2019; Vurukonda *et al*., 2018). En industria alimentaria, se emplean compuestos como natamicina (*S. natalensis*), siendo uno de los conservantes alimentarios más comunes que además puede ser empleado en diferentes alimentos como vinos, zumos, preparados cárnicos y quesos (Meena *et al*., 2021), o como la rubrolona (*S. echinoruber*), que produce un pigmento rojo empleado como colorante natural (Abraham *et al*., 2018; Sen *et al*., 2019).

Además de metabolitos secundarios, este género es productor de gran cantidad de enzimas interesantes en diferentes campos de la industria y que pueden llegar a sustituir compuestos químicos tóxicos y contaminantes. Entre algunos ejemplos, destacan las lipasas, empleadas para la fabricación de detergentes (Khushboo *et al*., 2022a). En el campo de la medicina, algunas enzimas se emplean como tratamientos frente a afecciones como la obesidad (lipstatina), o la trombosis (proteasa fibrinolítica); así como en pruebas diagnósticas (proteasa pronasa E o la fosfatasa estreptavidina) (Khushboo *et al*., 2022; Vojnovic *et al*., 2024). La azoreductasas son empleadas en combinación con otros

compuestos para la eliminación de colorantes azoicos, que, a pesar de ser altamente tóxicos y cancerígenos, son empleados por muchas industrias como la del papel, textil y cosmética (Khushboo *et al*., 2022). En la industria papelera también se usan xilanasas y lacasas para los procesos de deslignificación. En industria alimentaria y en la textil, se emplean xilasas, pectinasas y celulasas en detrimento de otros compuestos químicos que afectan al sistema nervioso y otros órganos. En el campo de la agricultura, muchos pesticidas pueden ser sustituidos por quitinasas como fungicidas e insecticidas (Kumar *et al*., 2020).

2.1.1-Ciclo de vida

Este género bacteriano se caracteriza por tener un ciclo de vida complejo basado en procesos de diferenciación y de muerte celular programada altamente ordenados, implicando enzimas específicas. Las esporas son formas de resistencia que permiten sobrevivir ante condiciones desfavorables de limitación de nutrientes. En un primer momento, estas germinan ante condiciones favorables, en un proceso ordenado y altamente regulado de oscurecimiento, hinchazón, y aparición del tubo germinativo (Yagüe *et al*., 2013). Este último se extiende por crecimiento polar y posteriormente ocurre la ramificación de las hifas (Jones *et al*., 2018).

En cultivos sólidos, este género presenta un ciclo de vida completo, comenzando a partir de la germinación de esporas y desarrollo posterior de las hifas, se forma un micelio compartimentalizado mediante septos membranosos (MI), muy diferentes estructuralmente a los septos que se formaran en las fases sucesivas. En esta estructura ocurren procesos de muerte celular programada en determinadas porciones (PCD), alternándose así segmentos vivos y muertos, donde las porciones muertas se lisan progresivamente (Manteca *et al*., 2006a; Manteca *et al*., 2006b). Las partes viables restantes sufren posteriormente un agrandamiento hasta generar un micelio multinucleado (MII), que es una estructura reproductiva que comienza a desarrollarse. El MII crece en el medio de cultivo y comienza a producir cubiertas hidrofóbicas (capa de chaplinas-rodlinas) para dar paso al crecimiento en vertical. En última instancia, este crecimiento aéreo reutiliza los nutrientes liberados por la etapa anterior durante un nuevo proceso de muerte celular programada, que culmina con la segmentación de las hifas para dar lugar

a la formación de cadenas de esporas (Manteca *et al*., 2010; Manteca *et al*., 2008). Las esporas son la forma de resistencia y dispersión, de manera que volverán a germinar ante condiciones favorables para iniciar un nuevo ciclo. El desarrollo del MII coincide con la fase de producción de metabolitos secundarios para evitar la competencia por otros microorganismos durante la esporulación (Manteca *et al*., 2008; Yagüe *et al*., 2013).

En cultivos líquidos, el desarrollo de *Streptomyces* es algo diferente, ya que solo unos pocos representantes de este género son capaces de esporular en este tipo de cultivo (Manteca *et al*., 2008). Esto es de potencial interés ya que la mayor parte de los cultivos a nivel industrial se llevan a cabo mediante fermentaciones en cultivo líquido. El ciclo de vida de los estreptomicetos en este tipo de cultivo mantiene la presencia y desarrollo de MI y MII sin capas hidrofóbicas, habiendo entre ellos una fase de detención del crecimiento donde los metabolitos son producidos por el micelio MII (Figura 2).

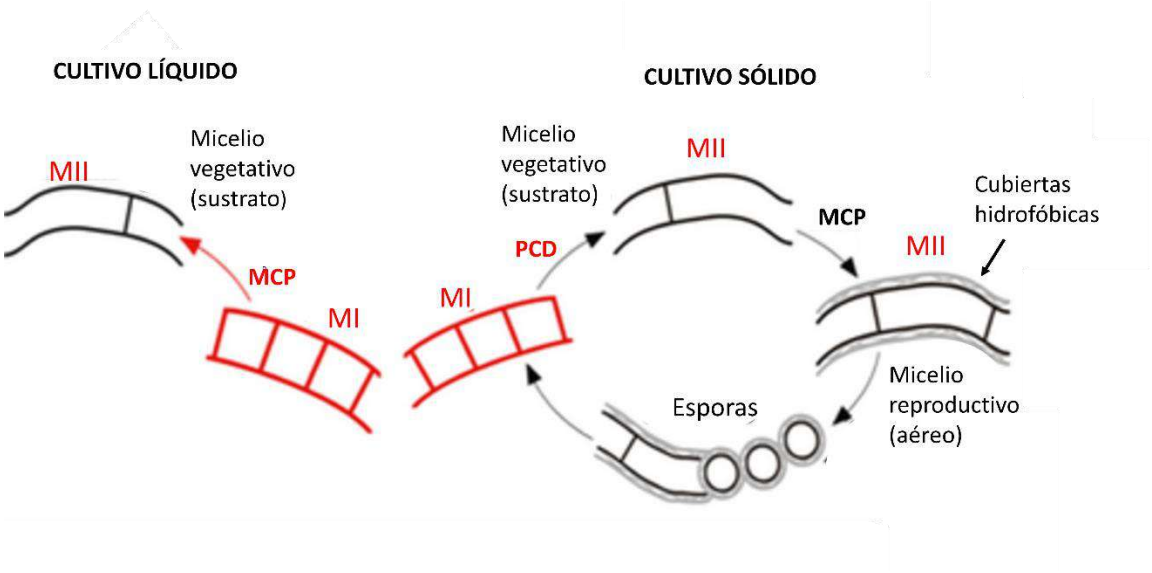


Figura 2: esquema del ciclo de desarrollo de *Streptomyces* sp. en cultivo líquido y sólido. Modificado de Yagüe *et al*., 2013. MCP = muerte celular programada, PCD = muerte celular programada, MI = micelio compartimentalizado, MII = micelio mutinucleado.

Este modelo de desarrollo ha sido recientemente descrito por Manteca y colaboradores (Manteca *et al*., 2008), donde en base a estudios de microscopia y transcriptómica demostraron la existencia del micelio MI, no incluido en el modelo clásico (Manteca *et al*., 2008; Manteca *et al*., 2009; Yagüe *et al*., 2013). El antiguo modelo (Figura 3) basaba el ciclo de vida de los estreptomicetos en la formación de un micelio sustrato y formación de micelio aéreo ante condiciones desfavorables o de

limitación de nutrientes, correspondiendo en el nuevo modelo con el micelio MII en fase temprana (sustrato) y fase tardía (aéreo) (Manteca *et al*., 2009; Yagüe *et al*., 2013).

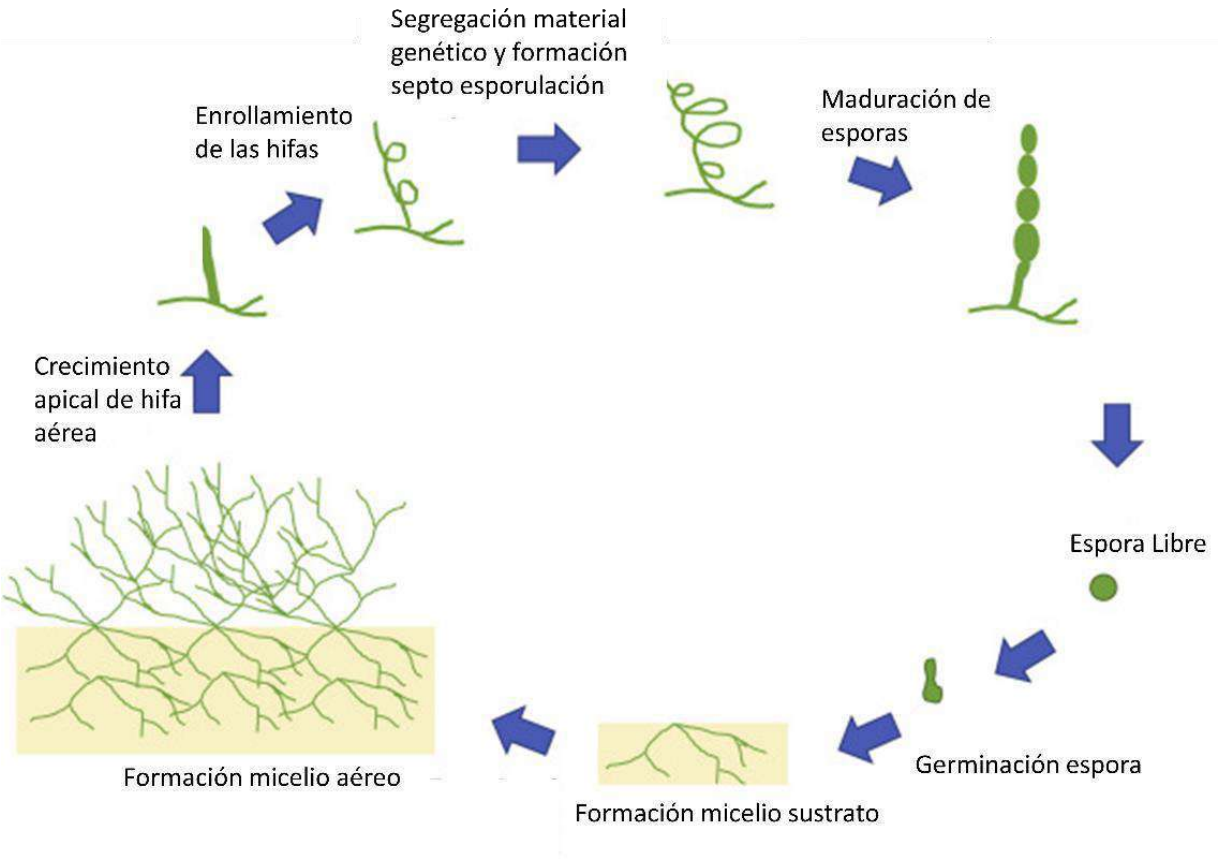


Figura 3: Modelo de desarrollo de *Streptomyces* clásico. Modificado de Hazarika *et al*., 2020.

2.1.2-Organización genómica

El ADN cromosómico de los miembros del género *Streptomyces* es lineal y de 8-10 Mb, un tamaño elevado en comparación con el de otras bacterias. Este factor, unido a su contenido en G+C de más del 70%, permiten diferenciarlo del de otros microorganismos (Omura *et al*., 2001). En la parte central (unas 6 Mb), se encuentran los genes esenciales, implicados en funciones vitales como la síntesis de macromoléculas, replicación, transcripción, traducción, mantenimiento y diferenciación celular; y que están conservados a lo largo de todo el género (Lee *et al*., 2020; Ventura *et al*., 2007). En los extremos se localizan grupos de genes menos conservados, entre los que se encuentran aquellos que forman parte del metabolismo secundario y son conocidos como

agrupaciones génicas biosintéticas (BGCs) (Figura 4) (Bentley *et al*., 2002). Esta zona está delimitada por secuencias invertidas repetidas (TIR), unidas covalentemente con las proteínas terminales. Estas estructuras son variables en secuencia y longitud según las diferentes especies (Lee *et al*., 2020).

Este genoma es muy plástico e inestable, es decir, mientras que la región central está muy conservada, las regiones de los extremos (similar a regiones subteloméricas) son muy variables y sensibles a modificación (Bury-Moné *et al*., 2023). Es frecuente que se produzcan deleciones, amplificaciones y reordenamientos espontáneos con frecuencias mayores de 0.1% y no solo en genes del metabolismo secundario, sino también en otros como los implicados en la diferenciación, formación del micelio aéreo, etc. En un primer momento, se consideró que esta inestabilidad se podía deber a la linealidad del cromosoma; sin embargo, se ha demostrado que mutantes con el cromosoma circular son aún más inestables (Pang *et al*., 2002; Volff *et al*., 1998).

Dada la diversidad de compuestos bioactivos producidos por los estreptomicetos, el estudio de su metabolismo secundario ha generado gran interés en la comunidad científica, dejando de lado el análisis de los genes del metabolismo primario que son los que proporcionan los precursores metabólicos. Sin embargo, ambos tipos del metabolismo están interconectados. Por ejemplo, ante un exceso de compuestos que no se puedan degradar, se activan vías del metabolismo secundario que utilicen dicho precursor (Hodgson, 2000) y viceversa, ante la necesidad de precursores para satisfacer la producción de metabolitos secundarios, se activan rutas del metabolismo primario para generar dicho precursor. Análisis del pangenoma de este género revelan que de media una especie puede llegar a codificar para la producción de unos 25-30 metabolitos secundarios, lo que pone de manifiesto el gran potencial biosintético de este grupo.

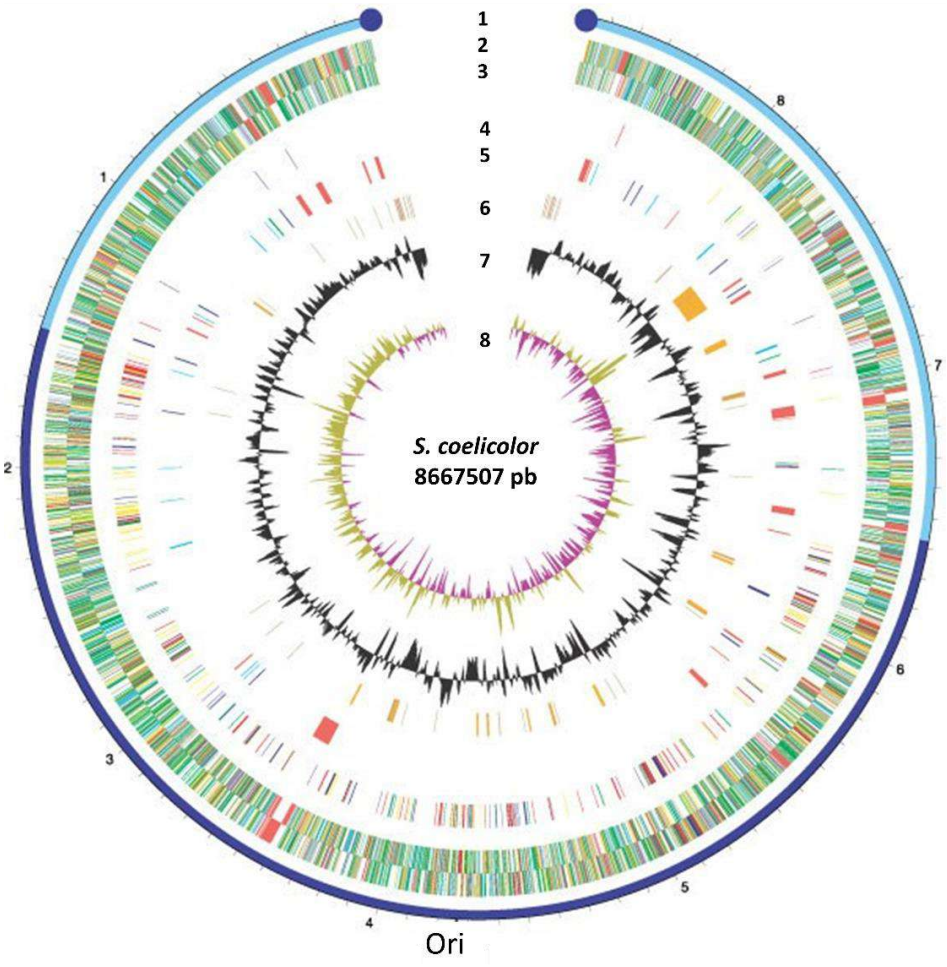


Figura 4: Representación del genoma de *Streptomyces coelicolor*. El circulo externo (nº1) en azul oscuro la región central del cromosoma, y en azul claro los brazos terminales. Se señala el origen de replicación (Ori) y las proteínas terminales al extremo. Los círculos nº 2 y 3 muestran todos los genes y su función, representándola con diferentes colores: negro para genes del metabolismo energético, rojo para transferencia de información y metabolismo secundario, verde oscuro para los asociados a la superficie, cian para la degradación de moléculas grandes, amarillo para el metabolismo primario o intermedio, magenta para la degradación de moléculas pequeñas, azul claro para los reguladores, naraja para genes hipotéticos conservados, marrón para pseudogenes, gris para miscelánea y verde claro para aquellos con función desconocida. El círculo nº 4 representa los genes implicados en funciones esenciales como la división celular, transcripción, traducción y replicación (mismo código de genes que el circulo anterior). El círculo 5 señala en rojo los genes del metabolismo secundario, en azul claro las exoenzimas, en verde las proteínas de vesículas de gas y en azul oscuro los conservones. El círculo 6 muestra en naranja los genes adquiridos por transferencia horizontal, y en marrón hacen referencia a los transposones. Por último, en el círculo 7 se representan el contenido en

G+C y el círculo 8 el sesgo GC, indicándose en morado los valores menores a uno y en marrón los mayores (Modificado de Bentley *et al*., 2002)

2.1.3-Metabolismo primario

El metabolismo primario es el encargado de regular reacciones indispensables, tanto anabólicas como catabólicas (Hodgson, 2000). Dentro de los genes del metabolismo primario, existen genes cuya expresión es estable o constitutiva y otros cuya expresión es variable.

Los genes conservados de mantenimiento (*housekeeping*) son aquellos esenciales para el mantenimiento celular y que se expresan de manera estable (Joshi *et al*., 2022). Entre los múltiples genes de este tipo, se mencionan los más relevantes para este trabajo:

- ***metK****:* Este gen codifica para la S-adenosilmetionina sintetasa que se encarga de catalizar la síntesis de S-adenosilmetionina (SAM) a partir de L-metionina y trifosfato de adenosina (ATP) (Zhang *et al*., 2008). SAM presenta un papel crucial actuando como donador de metilos a muchos compuestos del metabolismo primario y secundario, estando directamente implicada en la síntesis de metabolitos secundarios al inducir su producción (Yoon *et al*., 2006). Es por esto por lo que, la sobreexpresión de este gen en ciertas cepas de *Streptomyces* ha resultado en la sobreproducción de algunos metabolitos como actinorhodina (*S. coelicolor*), estreptomicina (*S. griseus*), avermictina (*S. avermitillis*) (Okamoto *et al*., 2003; Zhao *et al*., 2013).

- ***hrdB****:* codifica un factor sigma esencial, ya que su deleción es letal (Šmídová *et al*., 2019). Los factores sigma son subunidades multidominio de la RNA polimerasa bacteriana que presentan funciones clave en la iniciación de la transcripción (Paget, 2015). Este factor, no solo está implicado en la expresión de genes esenciales, sino que además interfiere en la diferenciación celular y el metabolismo secundario. El efecto que ejerce este factor sigma sobre el metabolismo secundario se basa en la iniciación de la transcripción de genes reguladores específicos de vías y a la mediación de flujo de energía y precursores entre el metabolismo primario y secundario. Debido a esta conexión entre los dos tipos de metabolismo, la manipulación del gen *hrdB* se utiliza

como estrategia para aumentar la producción de metabolitos secundarios (Sun *et al*., 2017).

- ***rpoB****:* Este gen codifica la subunidad β de la RNA polimerasa. Mutaciones en este gen activan rutas metabólicas silenciadas. Esto se puede deber a que la mutación mimetiza la unión de la RNA polimerasa con la alarmona ppGpp (nucleótido de guanina) implicada en la producción de metabolitos, activando así genes del metabolismo secundario (Hu *et al*., 2002). La alarmona ppGpp es un mensajero que media respuestas celulares de autoproteccion bajo estrés celular. En dependencia de la especie, varían los mecanismos por los cuales esta alarmona controla la transcripción y otros procesos metabólicos (Song *et al*., 2023).

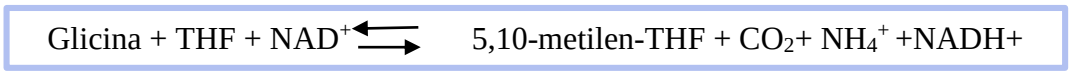
- ***rpsL****:* Codifica para la proteína ribosomal S12. Mutaciones en este gen, así como su sobreexpresión, se relacionan con activación de rutas metabólicas. Las mutaciones en este gen se producen en dos regiones conservadas dentro de la proteína S12 (Okamoto-Hosoya *et al*., 2003). Como la alarmona ppGpp es producida en el ribosoma, mutaciones en la proteína ribosomal pueden conducir a una gran producción de esta alarmona y como consecuencia a la activación del metabolismo secundario (Ochi *et al*., 2013).

- ***bldA:*** codifica un regulador de la diferenciación morfológica y la producción de metabolitos secundarios. Mutaciones en este gen en *S. coelicolor* dan lugar a un fenotipo carente de hifas y esporas, así como a la producción de antibióticos. Este efecto puede deberse al papel de *bldA* para codificar un ARNt único capaz de traducir el codón UUA. De esta manera, un gen con secuencia TTA solo puede traducirse en presencia de *bldA* funcional. Así mismo, este factor es también el responsable del efecto sobre la producción de antibióticos, ya que muchos genes que contienen TTA son reguladores que afectan a la transcripción tanto activándola como reprimiéndola. Se ha descrito que la expresión constitutiva de este gen causa activación de rutas silenciadas (Hackl *et al*., 2015)

En contraposición, otros genes del metabolismo primario se expresan dependiendo de la disponibilidad de nutrientes en el medio. Los estreptomicetos como organismos que fundamentalmente viven en el suelo, donde degradan la materia orgánica

de origen vegetal, presentan variedad de vías del metabolismo de los carbohidratos que están altamente reguladas y muchas de ellas son inducibles (Leblond *et al*., 1994). Del mismo modo, estos ambientes suelen ser competitivos y oligotróficos en nitrógeno y fosfatos y, por tanto, las vías del catabolismo de estos compuestos no son tan frecuentes. Por ello, se estima que la mitad de estas últimas, son inducibles y la otra mitad se encuentran expresadas a bajos niveles (Hodgson, 2000).

Dentro del grupo genes que se expresan en respuesta a cambios ambientales, y dada la relevancia de la producción de amonio para este trabajo, se desarrollará a continuación una breve explicación sobre el metabolismo de este compuesto. Esta vía es común tanto en plantas y animales, como en bacterias. La producción de amonio está determinada por el sistema de escisión de la glicina (GCV), que mediante una reacción de escisión oxidativa, que además es reversible, da lugar a CO_2, NH_4^+, y un grupo metileno aceptado por el tetrahidrofolato (THF) (Avalos *et al*., 2020).



Esta ruta metabólica consta de la acción de tres enzimas (GcvP, GcvT, GcvL) y una proteína transportadora (GcvH) (Avalos *et al*., 2020; Zhang *et al*., 2020). Inicialmente, GcvP descarboxila a la glicina liberando CO_2. Para esta reacción, GcvP precisa de GcvH, cuyo cofactor actúa como cosustrato. La parte restante de la molécula de glicina es transferida a uno de los grupos sulfhidrilo que se han generado por la escisión reductora del disulfuro en el lipoato que está unido a GcvH. Esta fracción unida a GcvH sufre una nueva degradación catalizada por GcvT en presencia de THF para dar lugar a amoniaco, N_5,N_{10}-metilen-THF y GcvH con el lipoato reducido. El último paso es la reoxidación del lipoato reducido unido a la proteína H y catalizado por GcvL (Figura 5). Además, el NADH producido se puede emplear posteriormente en reacciones de obtención de energía (Kikuchi *et al*., 2008; Xu *et al*., 2022). En *Streptomyces*, está descrito que, en la mayoría de los miembros de este grupo, la eliminación de *gcvP* o *gcvT* es suficiente para interrumpir la producción de amonio (Avalos *et al*., 2020).

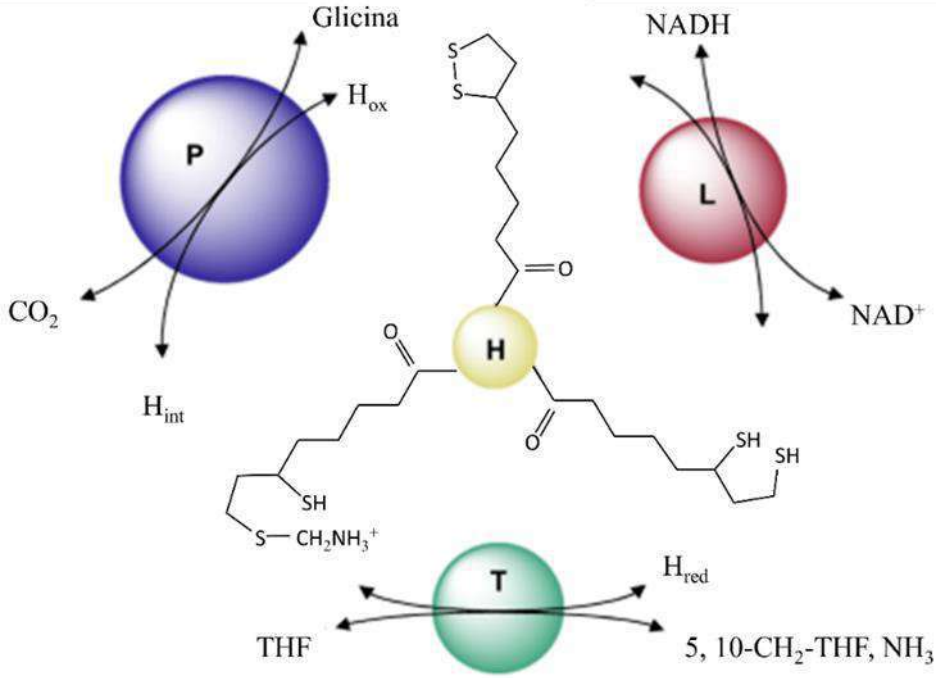


Figura 5: sistema de escisión de la glicina y reacciones catalizadas por cada proteína. THF= ácido tetrahidrofólico, H_{ox} = forma oxidada de GcvH, H_{int}=forma intermedia de GcvH, H_{red} =forma reducida de GcvH. Modificado de Zhang *et al*., 2020.

2.1.4-Metabolismo secundario

Como se ha comentado en apartados anteriores, el estudio del metabolismo secundario del género *Streptomyces* despierta gran interés entre la comunidad científica, dado su gran potencial para producir diversidad de metabolitos secundarios y muchos de ellos con propiedades bioactivas atractivas en diferentes sectores. La producción de estos compuestos está relacionada con la fase estacionaria de crecimiento del microorganismo. Este arsenal químico no es esencial; sin embargo, puede conferir ventajas sobre otros competidores (Sottorff *et al*., 2019). Los genes del metabolismo secundario se agrupan en BGC de manera que un grupo de genes microbianos actúan de manera coordinada y se encargan de la síntesis de uno o varios metabolitos secundarios (Crits-Christoph *et al*., 2021). Existen diferentes tipos de genes formando parte de estas agrupaciones:

- **Estructurales,** cuyos productos intervienen en la biosíntesis de un compuesto o en el ensamblaje de sus diferentes unidades. Los genes estructurales pueden

encargarse de la biosíntesis de un precursor del producto final no proporcionado por el metabolismo primario. Otros genes de este grupo se encargan de ensamblar distintos precursores para formar un núcleo estructural, como es el caso de policétido sintasas (PKS) o de sintetasas de péptidos no ribosomales (NRPS). Así mismo, existen genes que sintetizan elementos adheridos al núcleo estructural como pueden ser azúcares que contribuyen al aumento de la diversidad química y proporcionan las propiedades bioactivas de los productos naturales (Dickens *et al*., 1995; Olano *et al*., 2011).

- **Resistencia y transporte**, encargados de evitar los efectos nocivos que la producción de un compuesto pueda ejercer sobre el organismo que lo sintetiza. Pueden codificar proteínas de membrana que median la expulsión de compuestos en un mecanismo de intercambio acoplado a iones o protones, o transportadores de tipo ABC (transportadores dependientes de ATP); y también enzimas modificadoras del compuesto o de su diana (evitando así el daño en el organismo productor) (Olano *et al*., 2011; Tolba *et al*., 2006).

- **Reguladores,** controlan la expresión de los genes de biosíntesis y de resistencia, pudiendo ejercer efectos positivos o negativos sobre la expresión génica (Olano *et al*., 2011).

Los compuestos producidos por las agrupaciones de biosíntesis presentan gran diversidad estructural: policétidos, péptidos no ribosomales, terpenos, lactonas, quinonas heterocíclicas, indocarbazoles, péptidos de síntesis ribosomal modificados post-traduccionalmente (Ripps), sacáridos, alcaloides, etc. Se desarrolla brevemente a continuación la síntesis de los compuestos estructurales más representativos para este trabajo:

- **Policétidos:** Pertenecen a esta categoría la rapamicina (inmunosupresor producido por *Streptomyces hygroscopicus*), oleandomicina (antibiótico producido por *Streptomyces antibioticus*) o cromomicina A3 (antitumoral producido por *S. griseus*) (González-Sabín *et al*., 2012; Risdian *et al*., 2019). El proceso de biosíntesis es complejo, ya que requiere la participación de complejos multienzimáticos conocidos como

policétido sintasas (PKS). Estas PKS pueden ser de tipo I, II o III en función de la organización adoptada por las diferentes actividades participantes. El proceso comienza con una aciltransferasa (AT) que cataliza la unión de un acil-CoA a una proteína de unión de grupos acilo (ACP), y así sucesivamente se van uniendo residuos al ser condensados por una cetosintasa (KS). Las posteriores etapas son opcionales, lo que va a originar diferentes moléculas en función del grado de reducción ocurrido tras la condensación: cetoreductasa (KR, reduce el cetoéster), deshidratasa (DH, elimina el hidroxilo generado en el paso anterior y genera un doble enlace) y enoilreductasa (ER, reduce el enlace anterior convirtiéndole en sencillo).

En el caso de PKS de tipo I las diferentes actividades están organizadas en módulos dentro de una misma cadena polipeptídica, produciéndose en cada módulo un ciclo de condensación de forma no iterativa, de manera que se transfiere la cadena en formación de un módulo a otro hasta que se libera por acción de la tioesterasa (TE) final (Figura 6)(Risdian *et al*., 2019).

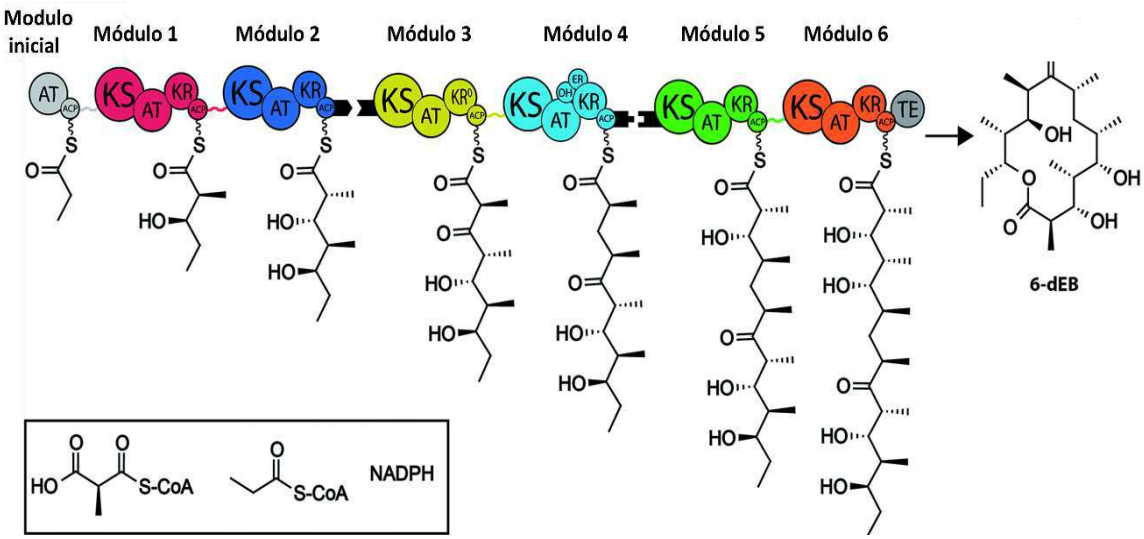


Figura 6: esquema de una policétido sintasa tipo I. Se observa como cada módulo contiene todos los elementos mínimos para llevar a cabo un ciclo de condensación. AT= aciltransferasa, ACP= proteína de unión de grupos acilo, KS=cetosintasaa, KR=cetoreductasa, DH= deshidratasa, ER=enoilreductasa, TE= tioesterasa. Modificado de Klaus *et al*., 2016.

Las enzimas PKS tipo II están encargadas de producir policétidos aromáticos, que se clasifican en 7 grupos: antraciclinas, anguciclinas, ácidos aureólicos, tetraciclinas, tetracenomicinas, polifenoles tipo pradimicina y benzoisocromanequiones. A diferencia

de las PKS tipo I, estas funcionan de manera iterativa con una única ACP sobre la que se construye la cadena con la participación conjunta de dos unidades de cetosintasa: KS_α se encarga de la condensación de precursores, mientras que KS_β determina la longitud de la cadena. Una vez formada esta estructura, pueden actuar las aromatasas, ciclasas y cetoreductasas para dar lugar al anillo aromático y, al igual que ocurre en las de tipo I, posteriormente otras enzimas pueden actuar para modificar estas moléculas (Figura 7).

Por último, las PKS tipo III, a diferencia de las de tipo I y II, no emplean ACPs como anclaje, sino que el acetil-CoA, y malonil-CoA para la extensión de la cadena son procesados directamente por una KS (Figura 7).

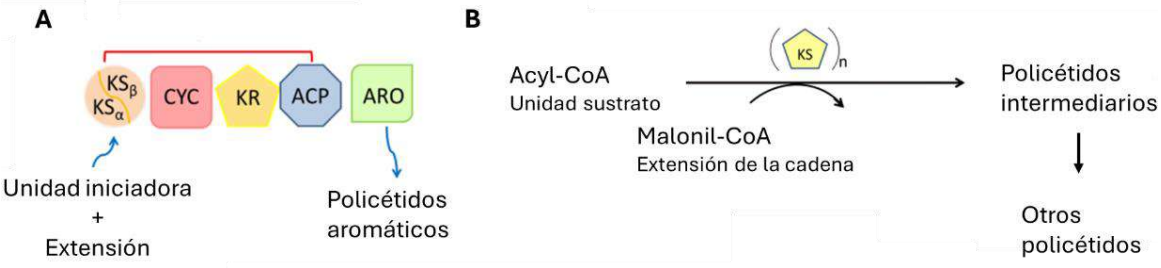


Figura 7: esquema de una policétido sintasa tipo II (A) y tipo III (B). KS=cetoacil sintasa, CYC= ciclasa, KR=cetoreductasa, ACP=proteína de unión de grupos acilo, ARO=aromatasa. Modificado de Wang *et al*., 2020.

❖ **Péptidos no ribosomales (NRP):** sintetizados por la actividad de NRPS que reclutan tanto aminoácidos proteinogénicos como no proteinogénicos e incluso ácidos grasos para construir la molécula peptídica. Las NRPS constan de un dominio de adenilación (A) que mediante su acción sobre un ácido carboxílico activa los aminoácidos a incorporar en la cadena. Este transfiere los sustratos como tioésteres covalentes al dominio de tiolación (T) sobre el que pueden producir modificaciones como metilación, formilación, epimerización oxidación, ciclación, o reducción, por parte de otros dominios. Posteriormente, el dominio de condensación (C) media la formación de enlaces peptídicos entre los aminoácidos incorporados por dos módulos adyacentes. Cuando la estructura molecular está terminada, es el dominio tioesterasa terminal (TE) el encargado de la liberación de este péptido, que puede mantenerse lineal o ciclarse por acción de ciclasas (Figura 8) (Xu *et al*., 2020). Como ejemplos de NRP producidos por

Streptomyces tenemos actinomicina D (antineoplásico, producida en este trabajo por *Streptomyces* CS131) o la daptomicina (antimicrobiano, producidos por *Streptomyces roseosporus*) (Liu *et al*., 2019; Miao *et al*., 2005).

Cabe destacar que existen metabolitos secundarios híbridos PK-NRP o NRP-PK (dependiendo de la orientación de sus dominios) como la bleomicina, antitumoral producida por *Streptomyces verticillus* (Chen *et al*., 2020; Courtial *et al*., 2022).

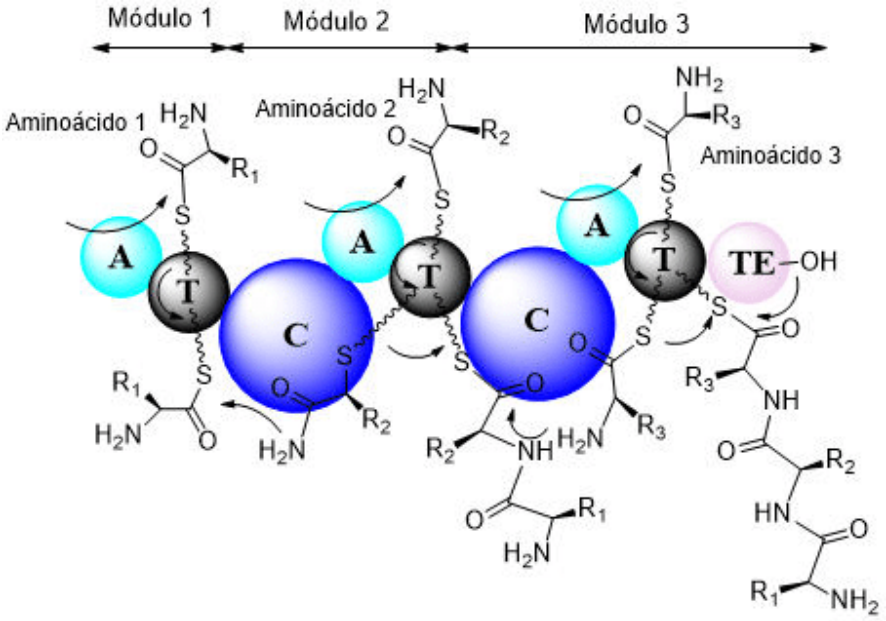


Figura 8: módulos básicos para la síntesis de una NRP. A=adenilación, T=tiolación, C=condensación, TE= tioesterasa. Modificado de González *et al*., 2016.

❖ **Terpenos:** Es el grupo de productos naturales de mayor diversidad estructural y actividades biológicas. Pueden ser volátiles o difusibles, y sufrir modificaciones diversas dando lugar a terpenoides, e incluso pueden generarse moléculas híbridas compuestas de terpenos y policétidos, alcaloides, aminoácidos, o fenol, dando lugar a los llamados meroterpenoides. Se clasifican en función del número de carbonos que contengan en monoterpenos (C10), sesquiterpenos (C15), diterpenos (C20), sesterterpenos (C25), triterpenos (C30), sesquarterpenos (C35) y tetraterpenos (C40). La habilidad de biosíntesis de estos compuestos ha sido descrita en plantas, hongos, cianobacterias, proteobacterias y actinobacterias (Tarasova *et al*., 2023). La síntesis de

terpenos se produce a partir de los precursores isopentenil difosfato (IPP) y dimetilalil difosfato (DMAPP), los cuales se pueden obtener por la vía del 2-C-metil-D-eritritol 4-fosfato (MEP) que es la más común en *Streptomyces*, aunque también se pueden producir por la vía del mevalonato (MVA).

La vía MVA consta de varias enzimas para la producción de IPP, actuando inicialmente la acetoacetil-CoA sintasa (AACS) si los precursores son malonil-CoA y acetil-CoA; o la acetoacetil-CoA tiolasa (AACT) si los precursores son dos moléculas de acetil-CoA (Dairi, 2005). A partir la secuencia de reacciones mostrada en la Figura 9 se obtiene IPP, que puede transformarse en DMAAP por acción de la IIP isomerasa (Takagi *et al*., 2000). Esta vía es común en mamíferos, hongos y en el citosol de las plantas.

Por otro lado, la vía MEP es la más común en los cloroplastos de las plantas, en la mayoría de las bacterias y en las algas verdes (Dairi, 2005). Esta ruta parte del piruvato y del D-gliceraldehido 3-fosfato (D-G3P) y el primer paso es catalizado por 1-desoxi-D-xilulosa 5-fosfato sintasa (DXS) que da lugar a 1-desoxi-D-xilulosa 5-fosfato (DXP). Posteriormente, actúa la enzima 1-desoxi-D-xilulosa 5-fosfato reductoisomerasa (DXR) dando lugar a 2-C-metil-D-eritritol 4-fosfato (MEP). Esta última molécula sufre posteriormente modificaciones y se activa mediante la acción de 2-C-metil-D-eritritol 4-fosfato citidiltransferasa (IspD), 4-(citidina 5-difosfo)-2-C-metil-D-eritritol cinasa (IspE) y de 2-C-metil-D-eritritol 2,4-ciclodifosfato sintasa (IspF). Finalmente, hay dos pasos de reducción catalizados por (E)-4- hidroxi-3-metilbut-2-enil-difosfato sintasa (IspG) y por 4-hidroxi-3-metilbut-2-enil difosfato reductasa (IspH) que dan lugar a IPP y DMAAP mediante reducciones catalizadas por los enzimas (E)-4- hidroxi-3-metilbut-2-enil-difosfato sintasa (IspG) y por 4-hidroxi-3-metilbut-2-enil difosfato reductasa (IspH), respectivamente (Xue *et al*., 2015) (Figura 9).

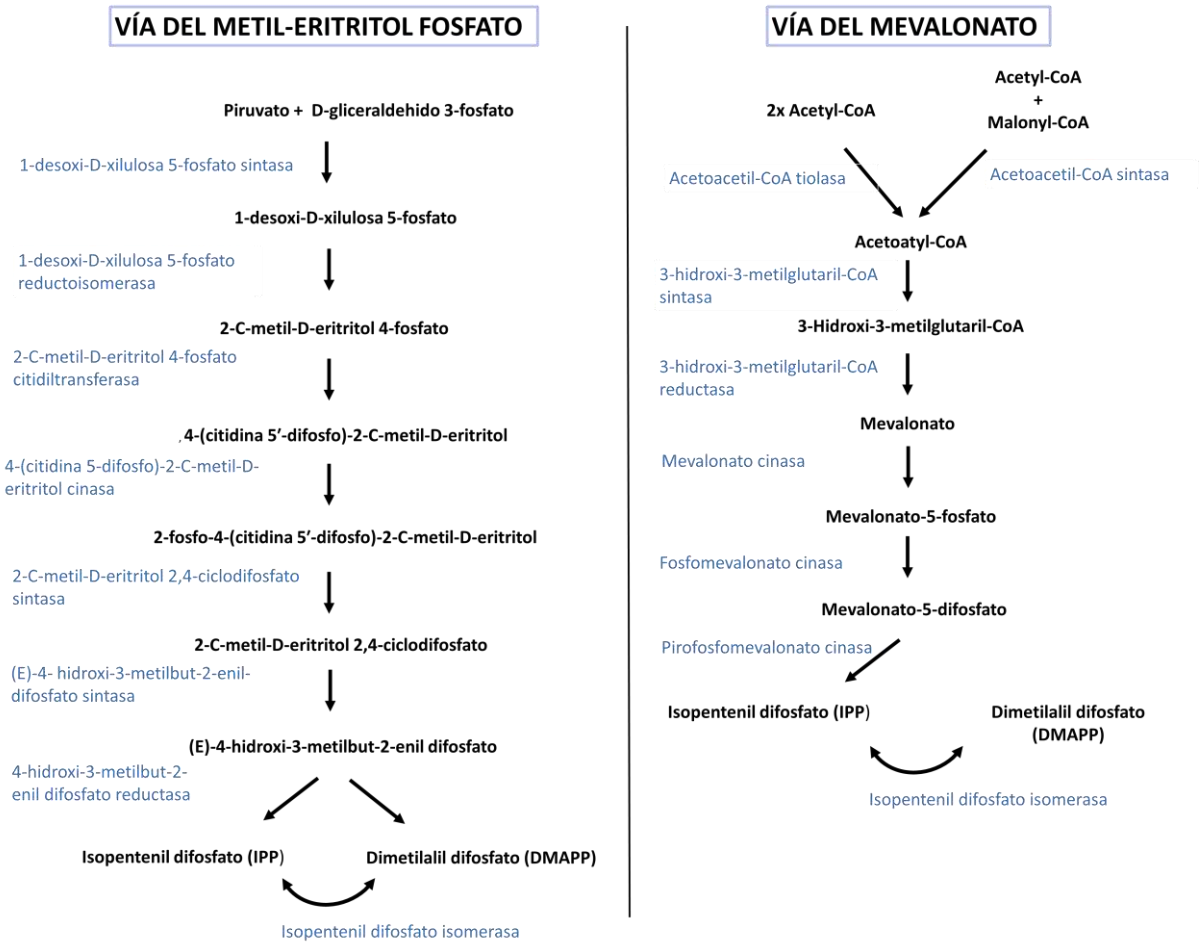


Figura 9: Vía del mevalonato y vía del metil-eritritol fosfato

Una vez obtenidos los precursores IPP y DMAAP, se generan diferentes residuos por adicción de IPP por las enzimas prenil transferasas: geranil pirofosfato (GPP), farnesil pirofosfato (FPP), geranil geranil pirofosfato (GGPP), geranil farnesil pirofostao (GFPP), etc. Posteriormente, las terpeno sintasas (TSs, también llamadas terpeno ciclasas) catalizan la ciclación de las cadenas hidrocarbonadas, dando así lugar a los diferentes terpenos (Figura 10).

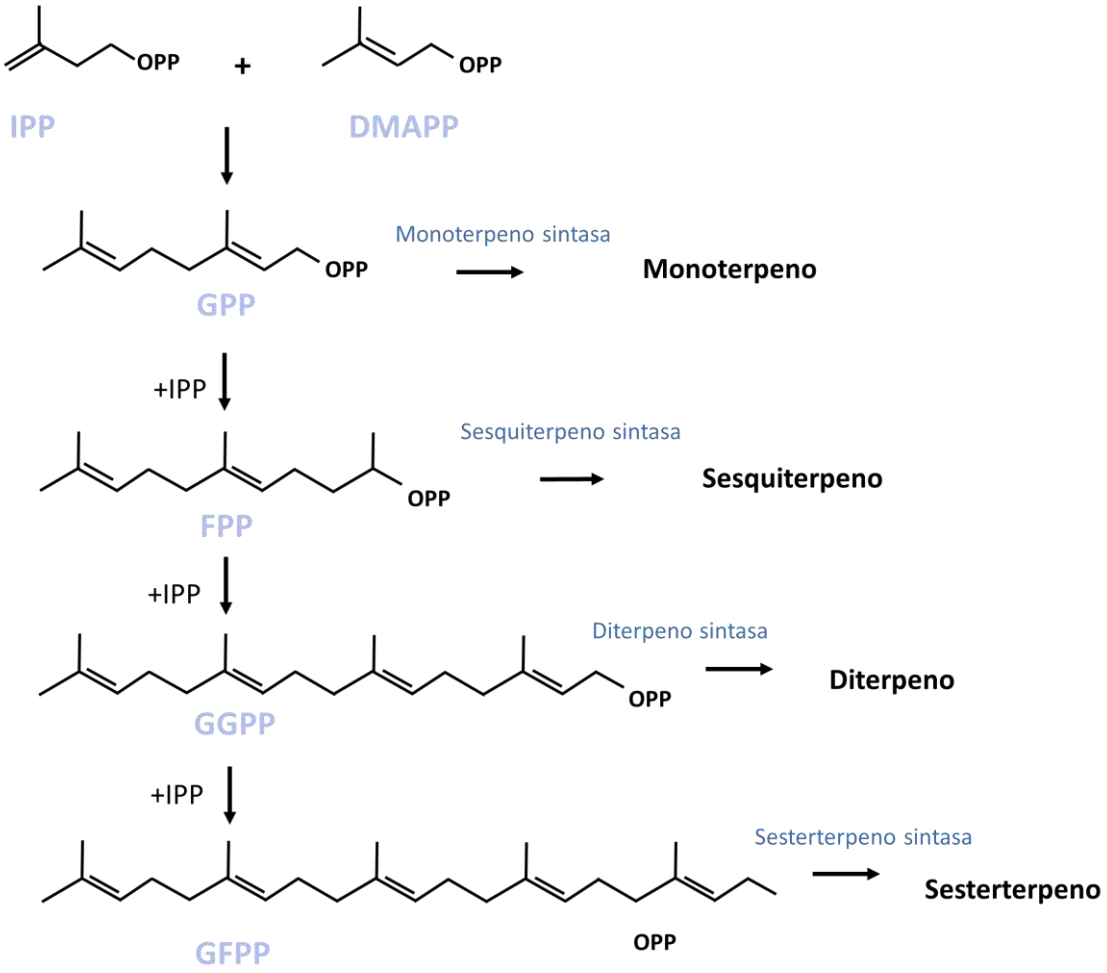


Figura 10: síntesis de diferentes terpenos. IPP = isopentenil difosfato, DMAAP= dimetilalil difosfato, GPP= geranil difosfato, FPP= farnesil difosfato, GGPP= geranilgeranil difosfato, GFPP= geranil farnesil difosfato.

2.1.5-Regulación del metabolismo secundario

Tal como se ha mencionado anteriormente, la producción de metabolitos secundarios en *Streptomyces* coincide o precede a la aparición de hifas aéreas en cultivo sólido, mientras que en cultivo líquido se limita a la fase estacionaria (Bibb, 2005). Uno de los puntos de control para iniciar el metabolismo secundario es la acumulación de N-acetilglucosamina (GlcNAc) por la degradación autolítica del micelio (Xia *et al*., 2020a). En general, los genes para la producción de metabolitos secundarios están agrupados en BGCs de extensión variable, pudiendo incluso alcanzar más de 100 kb. Muchos de estos

genes con funciones reguladoras son específicos de ruta, mientras que otros tienen funciones más globales. Por ejemplo, los genes *bld* están implicados en el metabolismo secundario, sin embargo, además están implicados en la formación de hifas aéreas y esporas (Bibb, 2005). La producción de metabolitos está regulada por cascadas transcripcionales con un control complejo y sujeto a diversas señales ambientales y fisiológicas (Kong *et al*., 2019), pudiendo clasificar la mediación de esta regulación en diferentes niveles:

2.1.5.1-Moléculas similares a hormonas y sus receptores acoplados

Son pequeñas moléculas difusibles, de bajo peso molecular, que interactúan con proteínas receptoras, que suelen pertenecer a la familia de los reguladores tipo TetR. Se las conoce como "hormonas de *Streptomyces"* y son responsables a pequeñas concentraciones (nanomolares) de iniciar cascadas reguladoras que pueden llegar a afectar en diferentes niveles jerárquicos, desde reguladores globales, hasta reguladores específicos de ruta y desembocar en la activación de rutas metabólicas (Kong *et al*., 2019). Se clasifican fundamentalmente en 5 tipos en función de sus características estructurales: γ-butirolactoras (en el 64% de los actinomicetos), γ-butenólidos (en el 24% de los actinomicetos), furanos, Factor PI y N-metilfenilalanil-deshidrobutirina dicetopiperazina (MDD) (Xia *et al*., 2020b). Dentro de esta clasificación las más importantes son las γ-butirolactoras y los γ-butenólidos, ambos basados en anillos heterocíclicos (Kong *et al*., 2019).

El factor A, una γ-butirolactona, es uno de los más estudiados y conservado dentro del grupo de los Actinomicetos. Este se une a su receptor ArpA y en *S. griseus* provoca una cascada de señales que da lugar a la producción de estreptomicina, grixazona, así como induce la diferenciación celular (Kong *et al*., 2019). Esta regulación es posible dado su efecto represor sobre el regulador pleiotrópico AdpA y el activador específico de grupo StrR, que activa todo el grupo de genes de biosíntesis de la ruta de estreptomicina (Horinouchi *et al*., 2007). Otro miembro de este grupo a destacar son los avenólidos de *Streptomyces avermitilis*, estructuras tipo γ −butenólidos, necesarios para la producción del antihelmíntico avermectina (Kitani *et al*., 2011).

2.1.5.2-Reguladores globales

Los reguladores globales tienen efectos pleiotrópicos, pudiendo afectar a más de una vía metabólica. Influyen en los procesos de diferenciación celular, en el metabolismo primario y secundario, así como responden a variedad de estímulos químicos como la limitación de nutrientes (Martín *et al*., 2012). Dentro de esta clasificación se encuentran los sistemas de dos componentes (TCS), que son los reguladores pleiotrópicos más abundantes, los cuales detectan estímulos ambientales e internos y los integran desencadenando respuestas celulares que permitan al organismo adaptarse a las condiciones cambiantes del ambiente (Sánchez de la Nieta *et al*., 2022). Estos mecanismos permiten el mantenimiento de la "homeostasis" en el metabolismo de diferentes vías. Los TCS constan de una histidina quinasa, un elemento sensor que percibe ciertas señales; y un elemento efector que regula respuestas celulares a estas señales. La detección de un estímulo por parte de la histidina quinasa provoca que se active y autofosforile en un residuo de histidina. Este fosforilo es transferido posteriormente a un grupo aspartato del elemento efector, generando su activación y una respuesta celular. A continuación, describimos brevemente algunos reguladores de este tipo que son relevantes en este trabajo:

- **DraR**: regulador global que actúa como activador en respuesta al exceso de nitrógeno (Jaishankar *et al*., 2017). Forma parte del sistema de dos componentes DraR-K y afecta tanto a la diferenciación morfológica y fisiológica, como a la regulación de la biosíntesis de metabolitos secundarios (Yu *et al*., 2012). En *S. coelicolor*, su deleción disminuye la producción de actinorhodina, aunque aumenta la producción de undecilprodigiosina así como un pigmento amarillo con estructura de policétido tipo I (Ceniceros *et al*., 2017; Yu *et al*., 2012).

- **Proteína receptora de AMP ciclico (Crp):** es un regulador muy conservado en bacterias, aunque ausente en *Bacillus* y otros Firmicutes**.** Presenta un papel crucial en el proceso de producción de antibióticos, desarrollo de colonias y germinación de las esporas, coordinando el flujo de precursores entre el metabolismo primario y el secundario (Xia *et al*., 2020b). La sobreexpresión de este gen conduce a una mayor producción de metabolitos secundarios, así como la producción de nuevos compuestos (Gao *et al*., 2012). En *S. coelicolor* conduce a la sobreproducción de undecilprodigiosina,

actinorhodina, y antibiótico dependiente de calcio. Así mismo, la eliminación de este gen conduce a la reducción de la producción de los dos últimos nombrados.

- **AbrC3**: forma parte de un sistema de dos componentes, siendo este el regulador de respuesta, mientras que consta de dos histidina quinasas (AbrC1 y AbrC2). La eliminación de este gen en *S. coelicolor* resulta en una disminución de la producción de metabolitos secundarios (actinorhodina y undecilprodigiosina) así como en un retraso morfológico, mientras que su sobreexpresión aumenta la producción de actinorhodina. Este gen presenta más de 20 ortólogos en otras especies de *Streptomyces* (Rico *et al*., 2014).

- **AfsR:** Forma parte del sistema de dos componentes AfsK/AfsR y regula positivamente el metabolismo secundario en *S. coelicolor* y la diferenciación celular en *S. griseus*. Cuando se activa AfsK, fosforila residuos de serina y de treonina de AfsR, aumentando su actividad de unión al ADN. Esta fosforilación, también puede ser realizada por otras quinasas. Este, también tiene un dominio de unión a ATP que determina su función ATP-asa, la cual es esencial para la activación transcripcional de *afsS*, su verdadera diana. *AfsS* codifica una pequeña proteína tipo factor sigma que actúa como activador específico de determinadas rutas metabólicas. La delección de *afsR* en *S. coelicolor* da lugar a la disminución de la producción de actinorhodina y undecilprodigiosina mientras que su sobreexpresión genera un aumento de producción de estos metabolitos, entre otros (Santos-Beneit *et al*., 2011).

- **BldD**: es un regulador de la transcripción que en *S. coelicolor* interviene en la producción de antibióticos, así como en el desarrollo morfológico. Muchas de las dianas de este regulador, codifican proteínas reguladoras, lo que resalta su papel como regulador global (Hengst *et al*., 2010). BldD es una proteína pequeña (18 kDa), con dos dominios, donde el dominio N-terminal media la unión al ADN. Entre varias dianas de este regulador, se encuentran dos factores sigma (σ^{BldN}, σ^{WhiG}) muy importantes para el desarrollo en *Streptomyces* y destaca el factor sigma σ^{H}, que media respuestas a estrés (Hengst *et al*., 2010). Mutantes en este gen en *S. coelicolor*, presentan un bloqueo en los primeros estadios de desarrollo morfológico, así como deficiencias en la producción de actinorhodina, undecilprodigiosina y antibiótico dependiente de calcio (Elliot *et al*., 1998).

2.1.5.3- Reguladores específicos de ruta (PSR)

Actúan como interruptores de la producción de metabolitos secundarios, ya que regulan directamente la transcripción de los genes estructurales y de resistencia presentes en las BGC; por ello, reciben el nombre de reguladores específicos de ruta (PSR). La mayoría de los genes reguladores de este tipo codifican activadores de bajo peso molecular (unos 25 kDA) (Martín *et al*., 2012). Entre ellos destacamos tres grupos: los SARP, LAL y la familia PAS-LuxR; aunque, los reguladores específicos de ruta pueden pertenecer a otras familias como es el caso de TetR, MarR, LysR e IclR (Xia *et al*., 2020a).

Los reguladores de tipo SARP (Proteínas Reguladoras de Antibióticos en *Streptomyces*) se caracterizan por presentar un dominio de unión a ADN tipo OmpR y un dominio de activación de la transcripción bacteriana (BTAD). A esta categoría pertenecen RedD y ActII-ORF4, activadores de la síntesis de undecilprodigiosina y actinorhodina respectivamente en *S. coelicolor* (Xia *et al*., 2020b).

El grupo de reguladores LAL (grandes reguladores de unión de ATP de la familia LuxR) generalmente actúan como activadores y presentan un dominio N-terminal de unión a ATP/GTP y un dominio C-terminal de unión a ADN de la familia LuxR. Reguladores como RapH, NysRI, AveR y SlnR controlan en *Streptomyces* la biosíntesis de diversos policétidos de tipo I (Xia *et al*., 2020a; Xia *et al*., 2020b).

Por último, los reguladores de la familia PAS-LuxR constan de un dominio sensorial PAS N-terminal que puede detectar señales internas (ya que se encuentran en el citosol) y otros factores ambientales; y un dominio de unión al ADN de tipo LuxR C-terminal. Un ejemplo de este tipo es PimR, que regula positivamente la producción de pimaricina en *S. natalensis*. Existen gran cantidad de ortólogos a *pimM* en otras agrupaciones de biosíntesis de compuestos antifúngicos (Xia *et al*., 2020a).

2.1.5.4-Regulación por retroalimentación

En este tipo de regulación, los productos finales o los intermediarios actúan como señales para regular la continuidad de la producción (Xia *et al*., 2020a). Así, en función de unas condiciones determinadas de cultivo, determinados compuestos como antibióticos actúan como ligandos, regulando así la producción de compuestos finales de una vía metabólica. Un ejemplo es la regulación de la producción de daunorrubicina,

donde el gen *DnrO*, que codifica una de las proteínas reguladoras implicadas en la biosíntesis de este compuesto, es reprimido por su propio producto mediante la interacción con el compuesto final de la ruta de biosíntesis (Jiang *et al*., 2006).

2.1.6-Importancia del estudio del metabolismo secundario

Desde la llamada "era dorada" del descubrimiento de productos naturales y el descenso del aislamiento de nuevos compuestos, se han tenido que explorar nuevas estrategias para tratar de identificar y caracterizar metabolitos no descritos. El desarrollo de técnicas de secuenciación masiva ha puesto en relieve el enorme potencial del grupo bacteriano de los estreptomicetos para producir metabolitos secundarios. Sin embargo, muchas de las agrupaciones biosintéticas que alberga su genoma (aproximadamente el 90%) se encuentran silenciadas en condiciones estándar de ensayo en el laboratorio, o son crípticas, desconociéndose el compuesto cuya producción determinan. Se estima que los genomas de los Actinomicetos albergan un gran número de agrupaciones (entre 18 y 37) implicadas en la biosíntesis de compuestos que no han sido identificados previamente (Olano *et al*., 2013; Ceniceros *et al*., 2021). Esto se traduce en que la mayoría de los productos de estas agrupaciones no se conocen y, por lo tanto, su caracterización despierta un gran interés en el campo de la investigación de metabolitos secundarios. Dada la creciente necesidad de nuevos compuestos naturales que puedan servir a nivel industrial por sus propiedades, desde hace un par de décadas se centran grandes esfuerzos en tratar de activar mediante diferentes estrategias estas agrupaciones génicas silenciadas (Liu *et al*., 2021).

Las estrategias de estudio de productos naturales microbianos pueden abarcarse desde una aproximación "top-down" basado en la metabolómica, donde en primer lugar se observa la producción de metabolitos para luego llevar a cabo un análisis posterior de los datos genómicos, o en sentido "down-top" donde gracias a la secuenciación de los genomas se pueden emplear diferentes herramientas bioinformáticas para así identificar agrupaciones silenciadas (Liu *et al*., 2021).

2.1.7- Herramientas para el estudio de agrupaciones de biosíntesis

La reorientación de las técnicas empleadas en detrimento de los métodos clásicos de aislamiento y caracterización, han permitido numerosos avances en este campo de investigación. Este flujo de trabajo requiere muchas veces de la aplicación integrada de diferentes estrategias y uso de diversas herramientas que, además de conducir al descubrimiento de compuestos, lo hagan de la manera más eficiente, economizando esfuerzos y costes. La aplicación de métodos de alto rendimiento (HTS) ha conducido a la identificación de gran variedad de moléculas, incluso se han aplicado métodos de química combinatoria para tratar de generar diversidad química (Albarano *et al*., 2020). Sin embargo, el proceso de descubrimiento también ha terminado por estancarse a pesar de estas aproximaciones, ya que es necesario ensayar gran cantidad de especies y de condiciones de activación, lo que reclama la necesidad de nuevas fuentes naturales para el aislamiento de posibles productores, así como de estrategias creativas.

2.1.7.1-Minería genómica

El concepto de minería genómica hace referencia a la identificación de agrupaciones génicas implicadas en la biosíntesis de productos naturales, a partir del análisis de la secuencia de los organismos productores, aportando información sobre las posibles interacciones funcionales y químicas de los genes y sus productos (Albarano *et al*., 2020). Esta tecnología es totalmente dependiente del uso de herramientas informáticas, tanto de aquellas encargadas de localizar las agrupaciones génicas como de aquellas que las comparan con la base de datos y ofrecen predicciones sobre sus funciones y posibles productos. Así, la minería genómica, junto al apoyo de herramientas como la manipulación genética, los métodos de interacción biológica, o las aproximaciones OSMAC (variación en las condiciones de cultivo), se ha convertido en una herramienta fundamental para el estudio de nuevos productos naturales y la caracterización de sus rutas biosintéticas. La minería genómica, además, debe tener un apoyo por parte de la biología sintética, de manera que se puedan construir circuitos genéticos que permitan descubrir nuevos productos naturales. Estos compuestos pueden sufrir modificaciones mediante el uso de diversas aproximaciones, así como puede mejorarse su producción (Figura 11) (Albarano *et al*., 2020; Olano *et al*., 2011).

Es de suma relevancia mencionar que, una vez identificado un grupo de genes se debe confirmar su implicación en la síntesis de un compuesto natural determinado. Esto se puede conseguir mediante diferentes estrategias como son la inactivación de genes en búsqueda de la interrupción de la producción de un compuesto, o la expresión heteróloga de los mismos en otro huésped que no lo produzca de forma natural (Olano *et al*., 2011).

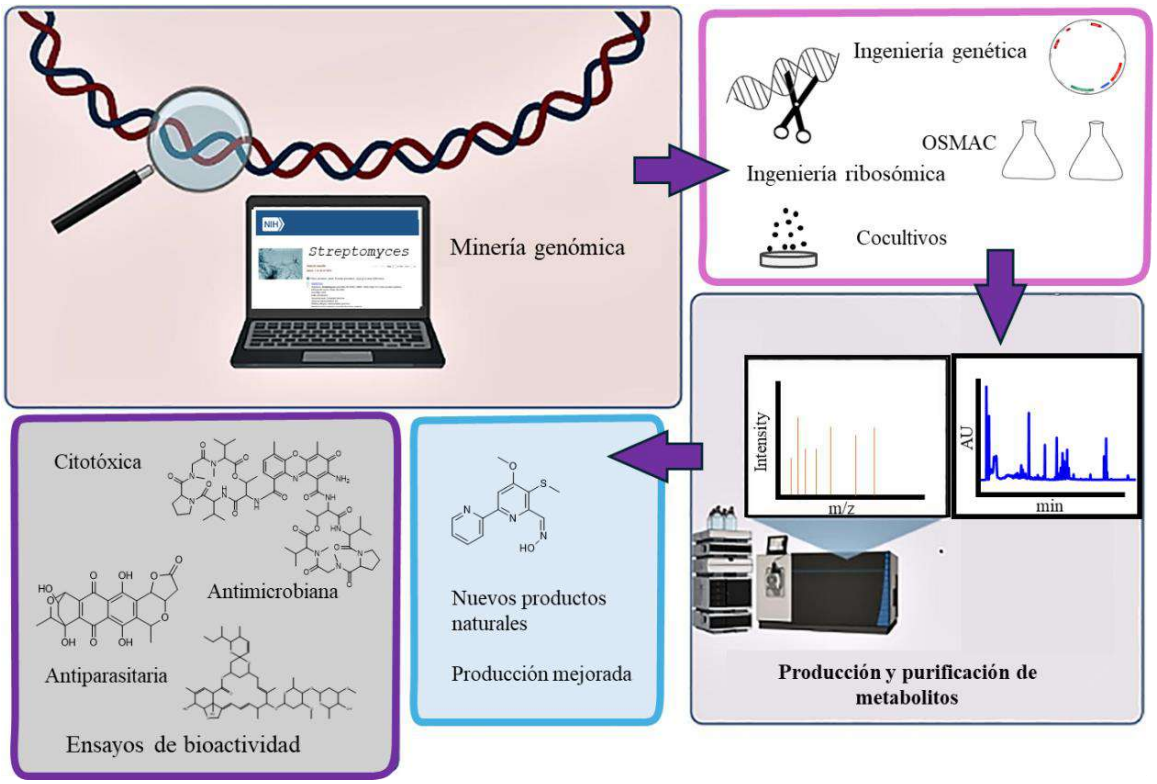


Figura 11: enfoque multidisciplinar e integrado a partir de estrategias de minería genómica en el proceso de descubrimiento de compuestos naturales. Modificado de Singh *et al*., 2021.

❖ Herramientas bioinformáticas

El empleo de herramientas bioinformáticas es fundamental en el proceso de descubrimiento de compuestos naturales. El uso, además, de métodos de análisis multiómicos, es clave para el estudio de la maquinaria biosintética de los organismos, permitiendo la identificación de los objetivos moleculares de los compuestos producidos (Ayon, 2023). Gracias a estas herramientas se puede predecir la localización de agrupaciones génicas de biosíntesis (BGCs) lo que supone de gran utilidad para tratar de

activar agrupaciones génicas silenciadas. Además, permite identificar BGCs que potencialmente puedan ser de interés, descartando otras ya conocidas o poco prometedoras, lo que además supone un ahorro económico, y en términos de esfuerzo y tiempo. Existen multitud de herramientas y *softwares* que facilitan esta tarea (Singh *et al*., 2022). El Centro Nacional de Información Biotecnológica (NCBI) ofrece gran variedad de información como son los datos de las secuencias de nucleótidos de diferentes especies depositadas en GeneBank®, permitiendo búsquedas de texto y descargas de información en diversos formatos (Sayers *et al*., 2022). Otro recurso indispensable es el empleo de BLAST (*Basic Local Aligment Search Tool)* que compara secuencias de nucleótidos o de proteínas con la base de datos, determinando similitudes a partir de análisis estadísticos (Wheeler *et al*., 2007). Por otro lado, la herramienta AntiSMASH, es una de las más empleadas en el análisis y estudio de metabolitos secundarios (Blin *et al*., 2023). Detecta y caracteriza BGCs tanto en genomas de hongos, bacterias y arqueas.

Adicionalmente, a partir de estos datos, se han creado otras herramientas de suma relevancia en el estudio metabólico, como puede ser ARTS2, buscador de genes de resistencia a antibióticos (Blin *et al*., 2023). Esta herramienta permite predecir el modo de acción de un compuesto producido por una agrupación no caracterizada en función de los genes de resistencia presentes en ella (Mungan *et al*., 2020). BiG-SLICE agrupa BGCs en familias mediante comparación con clústeres homólogos e identificando patrones que pueden relacionarse con funciones o metabolitos específicos. Esto permite clasificar agrupaciones de manera masiva, reconstruyendo un mapa global de la diversidad de metabolitos secundarios aún por explorar (Kautsar et al., 2021). El banco de datos MiBIG (*Minimum Information about Biosynthetic Gene Cluster*) ofrece información sobre agrupaciones ya descritas y su comparativa con otros clústeres similares (Terlouw *et al*., 2023). De manera similar funciona BiG-Fam, la cual es otra base de datos que identifica BGCs tanto estructural como funcionalmente en base a la comparativa con otras agrupaciones (Kautsar *et al*., 2021). ClusterFinder es una herramienta que, en base a modelos probabilísticos, identifica grupos de genes conocidos y desconocidos (Anker *et al*., 2024; Cimermancic *et al*., 2014). Estos son solo algunos ejemplos de la gran cantidad de algoritmos predictivos y bases de datos que se pueden emplear en el análisis bioinformático como soporte inicial para el estudio de la producción de productos naturales.

Una vez identificado el gen o los genes de la ruta biosintética objeto de estudio, se precisa el empleo de diferentes estrategias para tratar de caracterizar el compuesto determinado por dicha BGC e incluso modificarlo obteniendo nuevos derivados.

- **Manipulación genética, condiciones de cultivo e interacción biologica**

Existen diferentes aproximaciones que suponen modificaciones en el genoma (manipulación genética), así como estrategias basadas en la variación de condiciones de cultivo, y métodos de interacción biológica que pueden conducir a la activación de agrupaciones génicas silenciadas.

i) Expresión heteróloga: Se basa en la clonación del gen o genes en un vector de expresión y su introducción en un huésped heterólogo. De esta manera, se pueden clonar BGCs desconocidas en otros microorganismos para tratar de identificar los metabolitos producidos por dicha agrupación. En este contexto, se compararían los perfiles de producción del huésped heterólogo con y sin la expresión de nuevos genes. Es una herramienta útil y eficiente para tratar de activar rutas metabólicas, así como la expresión de genes optimizados (Liu *et al*., 2021). Ofrece la ventaja de elegir el nuevo huésped entre aquellos que presenten condiciones de crecimiento más favorables o conocidas. Se puede emplear segmentos nativos del organismo en cuestión o se puede utilizar segmentos biológicos sintéticos. Las estrategias más convencionales se basaban en el clonaje en cósmidos (BGCs < 40 kb), salvo en el caso del clonaje de BGCs de gran tamaño (>40 kb) que requieren construcción en BACs (cromosomas bacterianos artificiales). Esto último suponía un desafío técnico dado el gran tamaño de los fragmentos que los hace más vulnerables a cortes, así como reduce la eficiencia de transformación y ligación. Actualmente, se han desarrollado diversas tecnologías como CRISPR/Cas9 (repeticiones palindrómicas cortas, agrupadas y regularmente interespaciadas) o el ensamblaje de ADN que han permitido mejorar el tiempo y esfuerzo y de clonaje (Kang *et al*., 2021).

ii) Ingeniería de promotores: esta metodología resulta ser útil para tratar de activar la expresión de genes concretos o para aumentar su expresión. Se basa en sustituir los promotores nativos por otros promotores bien caracterizados, con expresión constitutiva (manteniendo niveles de expresión estables), inducibles (expresión ante

determinadas condiciones) y promotores con diferentes niveles de expresión (Xu *et al*., 2019). Un ejemplo es la activación de la producción de chatamicina B en *Streptomyces chattanoogensis* L10 como consecuencia de la sobreexpresión de un activador específico de ruta bajo la acción del promotor constitutivo *ermE** (Zhou *et al*., 2015).

iii) Deleción de genes represores y expresión de activadores transcripcionales: la deleción de genes con efecto represor en algunas ocasiones ha sido una aproximación exitosa en la activación de BGCs silenciosas, ya que libera su estado de bloqueo que se puede traducir en la producción de compuestos (Reen *et al*., 2015). Por ejemplo, la deleción del gen que codifica para el regulador global AdpA *en Streptomyces ansochromogenes* dio lugar a la activación del BGC de oviedomicina (Xu *et al*., 2017). Sin embargo, este mismo regulador, determina la expresión de nikomicina; por lo tanto, su deleción anula su producción (Liu *et al*., 2021). Esto indica que hay elementos reguladores que codifican para factores de transcripción, los cuales son clave para la activación de BGCs. En muchas ocasiones requieren para su activación de la unión a un ligando o a un cosustrato inductor que genera un cambio de conformación en los activadores, de manera que permite la activación de los promotores objetivo. Los miembros de las familias de los reguladores transcripcionales LysR y TetR suelen ser un objetivo común para la deleción, dado que su actividad es frecuentemente represora (Reen *et al*., 2015). Por otro lado, la sobreexpresión de activadores puede conducir a la activación de la producción de compuestos, así como al aumento de la producción de los mismos, como por ejemplo ocurre con la sobreexpresión de *slnR* en *Streptomyces albus* que da lugar a un incremento del 25% en la producción de salinomicina (Liu *et al*., 2021; Zhu *et al*., 2017).

iv) Ingeniería de ribosomas: este enfoque se basa en el empleo de microorganismos con la adquisición espontánea de mutaciones en el ribosoma o en la ARN polimerasa, mediante la detección de mutantes resistentes a determinados antibióticos. Esto conduce, en el mutante resistente, a la mejora de la producción de metabolitos secundarios, así como fomenta la activación de rutas metabólicas silenciadas**.** Este término se ha acuñado abarcando estas dos localizaciones posibles de las mutaciones (ARN polimerasa y ribosomas), a pesar de que las mutaciones en la ARN polimerasa, no sean estrictamente "ribosómicas" (Liu *et al*., 2021). Este sistema fue propuesto en 1996 por Dr. Ochi y colaboradores que determinaron que la adquisición espontánea de

resistencias a diferentes antibióticos daba lugar a mutaciones causando una desregulación en el metabolismo y consecuentemente afectando a la producción de compuestos naturales (Ochi *et al*., 2004; Shima *et al*., 1996). Así, las mutaciones en el gen *rpsL*, que codifica la proteína ribosómica S12, dan lugar a mutantes espontáneos resistentes a estreptomicina, lo que provoca un aumento de la alarmona ppGpp que tiene un papel crucial en la biosíntesis de antibióticos (Liu *et al*., 2021; Nguyen *et al*., 2020). Por otro lado, las mutaciones en el gen *rpoB*, que codifica la subunidad β de la ARN polimerasa, dan lugar a mutantes espontáneos resistentes a rifampicina, mimetizando la función de ppGpp unida a la ARN polimerasa y, por tanto, pudiendo conducir a la activación de segmentos silenciados o a la mejora de la producción (Liu *et al*., 2021; Nguyen *et al*., 2020). En concreto, se llevó a cabo un cribado con 1068 actinomicetos en el que se determinó que el 43 % de las cepas de *Streptomyces* sp. (51/119) no productoras de antibacterianos comenzaban a producirlos una vez adquiridas las resistencias a rifampicina o estreptomicina (Hosaka *et al*., 2009; Zhu *et al*., 2019). Además de estos antibióticos, la adquisición de resistencias a otros agentes como gentamicina, geneticina, ácido fusídico, tioestreptona, lincomicina, etc. han mostrado también la inducción de estos efectos sobre el metabolismo secundario (Zhu *et al*., 2019). La combinación de la adquisición de resistencias espontáneas a antibióticos ha mostrado ser causa de grandes cambios en el metabolismo, como en el caso de la cepa de *S. coelicolor*, que con la adquisición de resistencia a 7 antibióticos, la producción de actinorhodina aumentó 180 veces en comparación con la cepa silvestre (Wang *et al*., 2008).

v) Estrategia OSMAC: (una cepa, muchos compuestos) esta estrategia se basa en el cultivo de microorganismos en diferentes condiciones. La variación de parámetros como la temperatura, pH, composición del medio y uso de diferentes precursores puede inducir la activación de agrupaciones génicas silenciadas (Liu *et al*., 2021). Pequeños cambios en las condiciones de cultivo no solo pueden despertar la producción de nuevos compuestos, sino que puede mejorar sustancialmente la producción de otros metabolitos (Reen *et al*., 2015).

vi) Inducción de estrés: determinadas sustancias químicas pueden modificar el patrón de producción de metabolitos de los organismos. Al igual que la limitación de nutrientes supone una señal para iniciar la producción de metabolitos secundarios, otro tipo de estreses pueden llegar a desencadenar respuestas similares (Lejeune *et al*., 2022;

Reen *et al*., 2015). De este modo, se ha observado como la adición de antibióticos al medio de cultivo, la exposición al calor, a la congelación, cambios osmóticos o tratamientos con etanol suponen situaciones de estrés para los microorganismos lo que desencadena activaciones de rutas o una mejora en la producción (Ochi *et al*., 2013; Pickens *et al*., 2011; Reen *et al*., 2015).

vii) Elementos transponibles: son secuencias de ADN que pueden integrarse, copiarse y desplazarse a través del ADN cromosómico. Esto puede dar lugar a la modificación de la regulación en el genoma, ya que, dado su alta tasa de recombinación, pueden afectar a diferentes genes o movilizar genes adyacentes e introducirlos en el conjunto del ADN móvil (Lipszyc *et al*., 2022). De este modo, se pueden introducir promotores en localizaciones aleatorias, pudiendo llegar a activar genes latentes. Existen diversos sistemas de transposición, como puede ser el sistema Tn5, derivado de *Escherichia coli* (Lipszyc *et al*., 2022; Xu *et al*., 2017).

viii) Empleo de elicitores: Son pequeñas moléculas tanto bióticas como abióticas que pueden ejercer diversos efectos en el metabolismo, como activar rutas silenciadas o mejorar la producción endógena. Además, pueden ejercer otros efectos como inducción de la formación de biopelículas o modular la expresión de virulencia. Pueden ser fuentes nutricionales de carbono, nitrógeno o fósforo, moléculas microbianas como las ya mencionadas γ-butirolactonas o los γ-butenólidos, compuestos químicos como pueden ser las tierras raras o los metales pesados, DMSO, antibióticos, etc., y además, sus efectos pueden combinarse (Abdelmohsen *et al*., 2015).

ix) Señuelos de factores de transcripción: son segmentos de ADN de doble cadena que imitan el sitio de unión en el promotor de un factor de transcripción (Gambari, 2011). De esta manera, se interviene en la regulación dando lugar a la unión aberrante entre el factor de transcripción y el nuevo sitio de unión objetivo, lo que conduce a la disminución del efecto de represores o activadores, lo que en algunas ocasiones ha conducido a la activación de rutas silenciadas (Gambari, 2011; Hecker *et al*., 2017; Wang *et al*., 2019).

x) Cocultivos: el empleo de cocultivos puede ser ventajoso desde dos perspectivas. Por un lado, ciertos compuestos producidos por una cepa pueden ser

empleados como precursores por otras. Por otra parte, algunos compuestos generados por un organismo pueden actuar como agentes inductores de vías metabólicas silenciadas o mejorando la producción de las ya activas. Sin embargo, ciertos metabolitos también pueden actuar como agentes inhibidores de otros microorganismos al establecerse relaciones de lucha por competencia (Selegato *et al*., 2023). La comunicación entre organismos es clave para la activación o supresión de la producción de compuestos y a diferentes niveles (relaciones entre plantas, bacterias, hongos, animales) dado que la monitorización de las condiciones ambientales por parte de cada individuo puede desencadenar cambios del comportamiento y adaptar las actividades de transcripción y traducción en consecuencia (Reen *et al*., 2015). Esta aproximación trata de acercarse a la imitación de las condiciones ambientales en las que los microorganismos viven, pues son entornos complejos y cambiantes en los que dependen de gran cantidad de variables e interacciones.

xi) Biosíntesis combinatoria: Esta estrategia se basa en la modificación de rutas biosintéticas por medio de estrategias de ingeniería genética, de manera que, empleando la maquinaria biológica bacteriana, se puedan generar nuevos compuestos (Floss, 2006). El objetivo de esta estrategia radica en la generación de nuevos derivados y la obtención de rendimientos de producción mejorados, lo cual requiere del conocimiento de la estructura de la agrupación de estudio. Pueden emplearse diferentes aproximaciones como la eliminación de genes o la expresión heteróloga (comentadas en apartados previos), la biosíntesis dirigida por precursores o la mutasíntesis (Salas *et al*., 2009).

-**Biosíntesis dirigida por precursores:** donde compuestos no nativos son empleados como precursores de vías biosintéticas (Boddy *et al*., 2004).

-**Mutasíntesis**: biosíntesis dirigida por precursores en mutantes con etapas bloqueadas por la eliminación de genes (Olano *et al*., 2009).

Estas estrategias se pueden además combinar, de manera que la expresión heteróloga de genes se puede realizar en mutantes o en cepas productoras de compuestos en particular (Figura 12) (Olano *et al*., 2011). Una estrategia a destacar es la modificación

de enzimas condensadoras para generar compuestos con variaciones en el núcleo estructural (Olano *et al*., 2011; Sánchez *et al*., 2009).

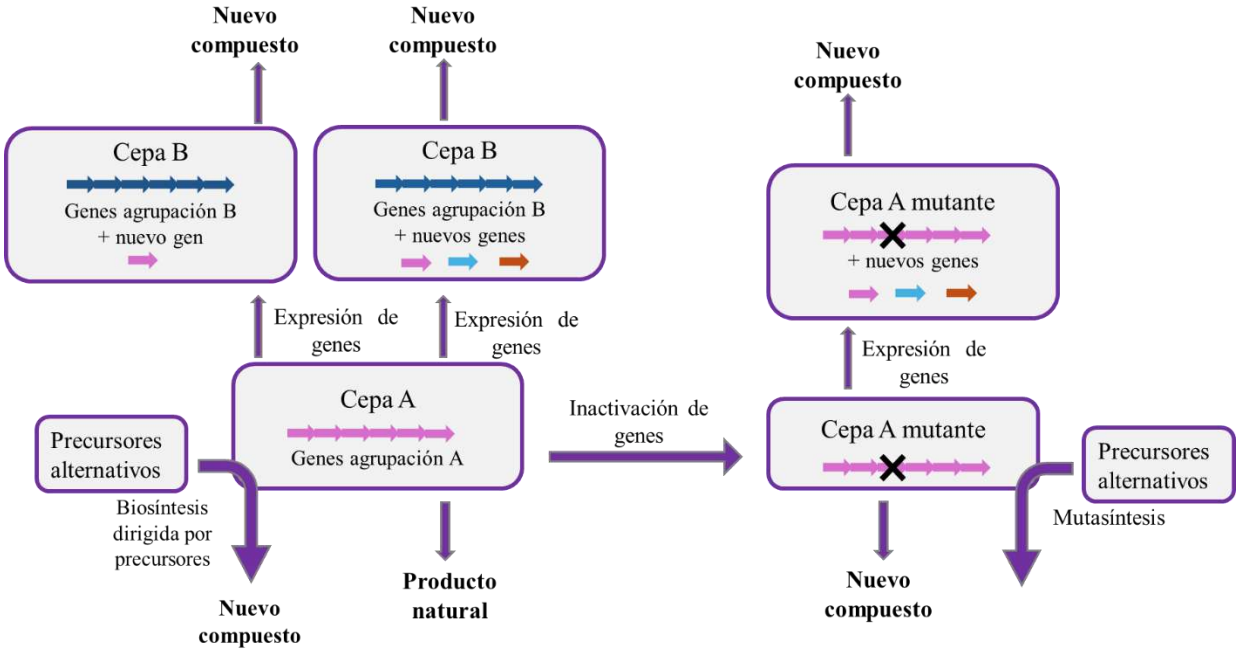


Figura 12: Estrategias de biosíntesis combinatoria que se pueden aplicar para generar nuevos derivados. Modificado de Olano *et al*., 2009.

2.1.7.2-Aislamiento de microorganismos en ambientes inhóspitos

Una estrategia eficiente en la búsqueda de nuevos compuestos naturales es el empleo de microorganismos aislados de ambientes poco convencionales o escasamente explorados, que pueden albergar agrupaciones genéticas de interés. Ambientes como entornos marinos, aguas profundas, desiertos, ambientes volcánicos o en asociación con otros organismos, son ejemplos de ambientes de vida con condiciones extremas a las que los microorganismos han tenido que adaptarse mediante modificaciones en su metabolismo (Sivalingam *et al*., 2019).

❖ **Asociación con otros organismos**

Es conocida la relación que mantienen algunos actinomicetos con otros organismos, ya que han sido descritas interacciones tanto con plantas, por parte de los géneros *Frankia* o *Micromonospora* (formadores de nódulos fijadores de nitrógenos en árboles), como con animales por *Pseudonocardia, Amycolatopsis y Saccharopolyspora* (mutualismo con insectos). Dentro del género *Streptomyces* sp. también han sido descritas interacciones tanto con vegetales, como con animales. Algunas especies son endofíticas y conviven en el interior de las plantas en una relación mutualista; o pueden localizarse en la rizosfera donde ejercer importantes papeles como promoción del crecimiento de la planta, desarrollo, tolerancia al estrés, así como inducción de resistencia a enfermedades, etc. (Quinn *et al*., 2020). Por otro lado, también se han descrito gran cantidad de interacciones de estos microorganismos con animales, en especial invertebrados. Se ha observado que ciertas bacterias establecen relaciones tanto transitorias como estables con esponjas marinas, y estas pueden ser de tipo mutualista o parasitario (Taylor *et al*., 2007). Los actinomicetos fundamentalmente en asociación con esponjas ejercen una función protectora sobre las últimas, y, además, los compuestos aislados de estos microorganismos han mostrado gran potencial bioactivo, en especial antibiótico frente a Gram negativos y Gram positivos (Cao *et al*., 2019; Quinn *et al*., 2020).

Los estreptomicetos también mantienen relaciones con insectos. Como ejemplo, está la relación mutualista endosimbionte que establecen con las avispas solitarias, que portan *Streptomyces* sp. en sus glándulas antenales, los cuales secretan metabolitos para proteger los nidos de las larvas de contaminación fúngica y bacteriana (Kaltenpoth *et al*., 2005). Otra asociación muy particular es la que establecen con hormigas cortadoras de hojas, que supone una relación esencial para los diferentes elementos implicados, garantizando el correcto balance y mantenimiento de dicho ecosistema.

o **Ecología de hormigas cortadoras de hojas. La colección CS.**

Las hormigas cortadoras de hojas (*Hymenoptera, Formicidae, Myrmicinae, Attini*) (Boulogne *et al*., 2014; Dhodary *et al*., 2021) son los miembros más representativos del grupo de las hormigas *Attini* (más de 250 especies) que comprenden los géneros *Att*a y

Acromymex. Se localizan fundamentalmente en las regiones de América Central y del Sur y representan el 25% de los herbívoros de los bosques de estas zonas. El ecosistema en el que viven es particular, ya que con sus excavaciones en el suelo alteran la aireación, y temperatura del mismo, así como mezclan capas de suelo más superficiales y ricas en materia orgánica con otras más profundas. Se estima que movilizan el 10-15 % de las hojas de las áreas circundantes para su alimentación. Sus nidos pueden llegar a tener extensiones de 50 m^2 y pueden alcanzar hasta 8 m de profundidad.

Estas hormigas cultivan hongos Basidiomicetos de la familia *Lepiotaceae*, *Leucoagaricus gongylophorus*, con los que conviven en una relación de simbiosis (Hölldobler *et al*., 1990). Este hongo degrada la materia vegetal recolectada por las hormigas para producir nódulos hifales sirviéndoles de alimento a los insectos, lo que les convierte en fungicultores, ya que dependen del hongo para la obtención de alimento (Dhodary *et al*., 2021). A cambio de este sustento, las hormigas le ofrece protección frente a otros microorganismos que puedan afectar a la estabilidad de la colonia, como pueden ser los patógenos *Fusarium* (*Hypocreales, Nectriaceae*), *Syncephalastrum* (*Mucorales, Syncephalastraceae*), *Trichoderma* (*Hypocreales: Hypocreaceae*) y en especial hongo patógeno especializado en este jardín, *Escovopsis weberi* (*Hypocreales: Hypocreaceae*), ya que mediante la producción de toxinas que degradan las hifas de los hongos basidiomicetos, amenazan con destruir las colonias de hormigas (Dhodary *et al*., 2018, 2021; Rodrigues *et al*., 2005).

Figura 13: hormiga *Atta echinatior* cubierta de actinomicetos en su superficie (Siavashifar *et al*., 2021).

Esta protección se la proporcionan a partir de los compuestos antimicrobianos producidos por los actinomicetos albergados en su superficie (Figura 13), entre los que destacan *Pseudonocardia* sp. y *Streptomyces* sp. Estos microorganismos presentes en el tegumento de las hormigas han coevolucionado para la producción de compuestos antifúngicos que protegen al hongo simbionte de la acción de los patógenos, participando así en una interacción multitrófica (Haeder *et al*., 2009). De este modo, cada uno de los miembros de este ambiente tiene un papel ecológico muy relevante

y su mantenimiento es esencial para el correcto balance de la estabilidad de los nidos de las hormigas.

En este trabajo, se utilizan diferentes cepas de *Streptomyces* de la colección CS, nombrada con las iniciales del Dr. Carlos Sialer, el cual se encargó de la recogida y aislamiento de los microorganismos a partir de hormigas cortadoras de hojas de la tribu *Attini* de la región de Lambayeque en Perú (Malmierca *et al*., 2018; Prado-Alonso *et al*., 2022).

3-Compuestos volátiles

Dentro de los metabolitos bacterianos, tanto primarios como secundarios, merecen una especial mención en este trabajo los compuestos volátiles (VCs). Dada la complejidad metabólica bacteriana, pueden pertenecer a diversidad de clases químicas, pudiendo ser productos del metabolismo primario, como pueden ser los productos de reacciones catabólicas (glicolisis, proteólisis, lipolisis, etc.), intermediarios de diferentes rutas o metabolitos secundarios (Alam *et al*., 2022; Audrain *et al*., 2015). Pueden ser moléculas inorgánicas u orgánicas, estos últimos conocidos como compuestos orgánicos volátiles (VOCs). Los VOCs son pequeñas moléculas que pueden presentar gran diversidad estructural, aunque mantienen una serie de características en común: son moléculas de bajo peso molecular (<300 Da), alta presión de vapor (0.01 kPa a 20 ºC), bajo punto de ebullición, y naturaleza lipófila (Audrain *et al*., 2015; Cellini *et al*., 2021; He *et al*., 2022). Esto hace que difundan fácilmente tanto en agua como en aire, lo que los convierte en mensajeros químicos de acción rápida (Schulz-Bohm *et al*., 2017).

Al igual que cualquier otro tipo de metabolitos, la producción de estos compuestos es dependiente de las condiciones de cultivo: sustrato, temperatura, humedad, estado de crecimiento y desarrollo, disponibilidad de nutrientes, etc. (Inamdar *et al*., 2020); dando lugar a mezclas complejas que constituyen el volatiloma de una especie o grupo en particular (Cellini *et al*., 2021). A pesar de que se desconocen gran parte de los compuestos volátiles producidos por bacterias, algunos de ellos son característicos de un grupo bacteriano en concreto, lo que constituye una firma metabólica útil en la identificación de microorganismos (Netzker, 2020).

En los últimos años, el estudio de estos metabolitos ha alcanzado gran relevancia debido a la diversidad de propiedades bioactivas asociadas a los mismos, así como la importante función ecológica que presentan como moléculas de señalización a nivel tanto interespecífico como intraespecífico. Esto permite la comunicación entre los organismos más simples hasta los más complejos, incluso sin necesidad de producirse contacto físico entre los mismos, lo que pone en relieve la complejidad de interacciones que median estos compuestos en un ecosistema determinado (Schulz-Bohm *et al*., 2017; Tyagi *et al*., 2020). Así, la emisión de VCs puede actuar como señal de defensa, como repuestas de competición, de cooperación, de agregación, cambios en motilidad, formación de biopelículas, de inducción de factores de virulencia o resistencias, inductoras del crecimiento o de la producción de compuestos, inhibición del crecimiento, cambios en la capacidad de esporulación, cambios en la permeabilidad y fluidez de membrana (Cellini *et al*., 2021; Khushboo *et al*., 2022; Naik *et al*., 2023).

Los compuestos volátiles microbianos (MVCs) son una de las principales vías de comunicación entre bacterias, así como entre bacterias y plantas o animales, y la producción de determinados compuestos puede determinar cambios de comportamiento y funciones en comunidades complejas. Esta vía de comunicación permite ejercer efectos sobre los organismos vecinos de manera rápida y a una distancia mayor de la que pueden alcanzar los compuestos difusibles (Sharifi-Rad *et al*., 2015).

El estudio de este tipo de moléculas ha permanecido en un segundo plano frente al estudio de los compuestos difusibles, dada la gran dificultad de estudio de los mismos por su naturaleza gaseosa, y la falta de métodos de análisis estandarizados. En los últimos años, se han desarrollado gran cantidad de métodos de estudio de VCs basados en análisis por GC-MS. Lo más común, es trabajar con mezclas complejas de compuestos a bajas concentraciones, por lo que la fase de colección de muestras es clave, y es importante evitar la contaminación con compuestos que se encuentren en el ambiente (Vlot *et al*., 2022; Weisskopf *et al*., 2021). Existen diferentes métodos de recolección de compuestos volátiles, así como de análisis de los mismos y estos deben adecuarse al estudio que se realice, por lo que es importante seguir una metodología adaptada al tipo de muestra y el objetivo de análisis (Cuervo *et al*., 2024). A pesar de estas dificultades, se pueden identificar incluso cuantificar compuestos aislados a partir de muestras complejas.

El estudio de estos compuestos se encuentra en auge ya que presentan gran cantidad de propiedades de interés que son empleadas y explotadas a nivel industrial. En cuanto al género *Streptomyces*, el gran potencial metabólico que presenta también se exhibe en su volatiloma, en el que coexisten moléculas con propiedades antifúngicas, antivirales, antibacterianas, etc., así como moléculas de señalización claves para la comunicación y coordinación en ambiente en el que se encuentran.

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II. Objetivos

El objetivo de este trabajo está enfocado en la aplicación de diferentes herramientas de control genético y aproximaciones basadas en la interacción biológica para mejorar la producción de compuestos, así como para activar rutas metabólicas silenciadas en *Streptomyces* spp. Para lograr este propósito, este proyecto se divide en los siguientes apartados:

- Mejora y activación de la producción de compuestos mediante expresión heteróloga de reguladores globales y genes *housekeeping* descritos como responsables de activación o sobreproducción de compuestos, en combinación con la aproximación OSMAC y con la obtención de mutantes por ingeniería ribosómica.

- Evaluación del efecto sobre el metabolismo y la capacidad de crecimiento de diferentes cepas de la colección CS ante la interacción biológica mediada por compuestos volátiles

- Análisis de las propiedades antifúngicas de los compuestos volátiles producidos por diferentes cepas de *Streptomyces* spp. y evaluación a nivel ecológico de la implicación de estos compuestos en los nidos de las hormigas cortadoras de hojas.

III. Resultados

Capítulo I

Co-Expression of Transcriptional Regulators and Housekeeping Genes in *Streptomyces* spp.: A Strategy to Optimize Metabolite Production

microorganisms

MDPI

Article

Co-Expression of Transcriptional Regulators and Housekeeping Genes in *Streptomyces* spp.: A Strategy to Optimize Metabolite Production

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Abstract: The search for novel bioactive compounds to overcome resistance to current therapeutics has become of utmost importance. *Streptomyces* spp. are one of the main sources of bioactive compounds currently used in medicine. In this work, five different global transcriptional regulators and five housekeeping genes, known to induce the activation or overproduction of secondary metabolites in *Streptomyces coelicolor*, were cloned in two separated constructs and expressed in 12 different strains of *Streptomyces* spp. from the in-house CS collection. These recombinant plasmids were also inserted into streptomycin and rifampicin resistant *Streptomyces* strains (mutations known to enhance secondary metabolism in *Streptomyces*). Different media with diverse carbon and nitrogen sources were selected to assess the strains' metabolite production. Cultures were then extracted with different organic solvents and analysed to search for changes in their production profiles. An overproduction of metabolites already known to be produced by the biosynthesis wild-type strains was observed such as germicidin by CS113, collismycins by CS149 and CS014, or colibrimycins by CS147. Additionally, the activation of some compounds such as alteramides in CS090a pSETxkBM-RRH and CS065a pSETxkDCABA or inhibition of the biosynthesis of chromomycins in CS065a in pSETxkDCABA when grown in SM10 was demonstrated. Therefore, these genetic constructs are a relatively simple tool to manipulate *Streptomyces* metabolism and explore their wide secondary metabolites production potential.

Keywords: *Streptomyces*; global regulators; overproduction; heterologous expression

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1. Introduction

The current growing resistance to antibiotics due to the misuse of drugs, as well as their abusive use in livestock, threatens to be one of the main causes of death in the near future. The SARS-CoV-2 pandemic has culminated, among many other effects, in the acceleration of antibiotic resistance due to antibiotic prescriptions given to SARS-CoV-2-infected patients to prevent secondary bacterial infections [1,2]. It is urgent to find new treatments against new diseases as well as to improve existing ones. Different strategies are followed to avoid resistance mechanisms, such as combining different compounds that have a synergic activity, chemical modification of current compounds to alter their activity, looking for novel bioactive compounds produced by microorganisms isolated from underexplored environments, or the use of bacteriophages, among others [3–9].

Natural products have long provided the active principle for many drugs due to their enormous structural and chemical diversity. However, after the golden era of drug discovery in the 1950s, it is increasingly challenging to find new metabolites of interest. Improvement in sequencing techniques and bioinformatics analysis has shown that microorganisms have the potential to produce many unknown secondary metabolites that could

potentially have novel structures, which could avoid current resistance mechanisms [8–11]. However, despite the wide array of -omic technologies currently available, the continuous rediscovery of already known compounds together with the technical limitations for detecting compounds is increasing the difficulties of finding novel products. Furthermore, it is not always possible to obtain a sufficient level of production by the microorganism to make the compound attractive for industrial-scale manufacturing. Many compounds are produced under very specific conditions, which are difficult to reproduce in the laboratory. On some occasions, the gene clusters responsible for the biosynthesis of these metabolites might not be complete or an unknown regulator is repressing its expression [12,13]. In other cases, precursors might not be available for biosynthesis or are being exhausted by other biosynthesis pathways. Many strategies have been developed to overcome these problems, such as redirecting precursors to the target biosynthesis by disrupting or deleting highly active routes that may be consuming precursors, testing different production media and conditions, heterologous expressing genes that seem to be missing or defective, or regulators expected to activate the pathways [12–15].

There are many successful examples of transcriptional regulator engineering applied to the activation of secondary metabolites. In 2018, Guo and co-workers improved avermectin production by *S. avermitilis* by deciphering the regulatory cascade involving the SAV4189 pathway-specific transcriptional activator [16]. However, the engineering of global regulators can also be used to modify the production of metabolites of interest, as was demonstrated by the activation of nikkomycin biosynthesis by the disruption of the *adpA* gene in *Streptomyces ansochromogenes* [17]. Previous studies have shown how the introduction or deletion of several regulators at the same time generates different synergistic effects on the recipient strain [18].

Ribosome engineering is a strategy that relies on the discovery of strains with mutations in their ribosome or RNA polymerase (through screening of streptomycin and rifampicin resistant mutants), resulting in the enhancement of enzyme production. Analysis of the metabolite profile of these resistant strains showed the production of metabolites that are undetectable in wild-type strains [19,20].

As mentioned above, one interesting approach for new drug discovery is to explore the biosynthetic potential of microorganisms that live in underexplored ecosystems or symbiosis with other organisms. Insect microbiota has recently drawn attention for its antifungal and antimicrobial activities [17,18]. The Carlos Sialer collection (CS), isolated from the tegument of ants from the Attini tribe, was recently analysed as a source of novel secondary metabolites [19,20].

In this work, we followed a multi-angle strategy for the modification of the secondary metabolism of twelve *Streptomyces* strains from the CS collection. Two different vectors were generated and introduced in the wild-type CS strains: one containing five housekeeping genes (pSETxkBMRRH) and another containing five regulatory genes (pSETxkDCABA). These genes were carefully selected based on their characteristics, and their involvement in the regulation of secondary metabolism and the production of bioactive compounds (Tables 1 and 2). It was also estimated that the introduction of a battery of genes will generate a response that may not be generated with the introduction of these genes separately, since it is well known that regulators interact with each other [21–24].

Table 1. Global regulators and their implication on *Streptomyces* regulation. ACT (actinorhodin), RED (undecylprodigiosin), CDA (calcium-dependent antibiotic), and yCPK (yellow-pigmented secondary metabolite). ↓↓ Indicates a decrease in production and ↑↑ indicates an increase in production.

Gene Product	Influence in *Streptomyces* Secondary Metabolism	Effect of Deletion in *S. coelicolor*	Effect of Overexpression in *S. coelicolor*	References
CRP (*Cyclic AMP receptor protein*)	Regulation of the synthesis of several antibiotics	↓↓ ACT and CDA	↑↑ ACT, CDA and RED	[25]
AfsR	Pleiotropic regulatory protein for antibiotic production	↓↓ ACT and RED	Increase metabolite production ↑↑ ACT and RED	[26–28]
BldD	Control of morphological development and antibiotic production	↓↓ ACT, RED, and CDA. Deficiency in sporulation and antibiotic production	Not found	[29,30]
DraR	Activation/inhibition of antibiotic production	↑↑ yCPK, ↓↓ ACT	Not found	[26,31]
AbrC3	Activation of antibiotic production	↓↓ ACT and RED	↑↑ ACT	[32]

Table 2. Housekeeping genes and their effects on the metabolism of *Streptomyces* spp.

Gene	Gene Product	Effect on Antibiotic Production in *Streptomyces* spp.	References
metK	S-adenosylmethionine synthetase	Overproduction of ACT in *S. coelicolor* and streptomycin in *S. griseus* and 2-to-5-fold higher production of avermictin in *S. avermitillis.*	[33,34]
hrdB	RNA polymerase main sigma factor. Binds promoters of different secondary metabolism clusters	Induced expression of the gene showed increased promoter activity in *S. coelicolor*	[35,36]
rpoB	RNA polymerase β subunit	A point mutation in the gene activates antibiotic production in *S. lividans.* No information was found about its overexpression.	[37]
rpsL	Ribosomal protein S12	Point mutation in the gene resulted in an overproduction of antibiotics in *S. coelicolor*. Overexpression of wild-type *rpsL* also showed the activation of compounds in *S. clavuligerus*	[37,38]
bdlA	tRNA translates UUA codon to leucine	Overexpression of the gene in *S. coelicolor* activates the expression of silent clusters	[39]

In addition, streptomycin and rifampicin resistant strains were generated and their metabolic profiles were analysed after the introduction of the aforementioned vectors. This work aims to evaluate the effect of these housekeeping and global regulators genes when heterologously expressed in different *Streptomyces* strains.

2. Materials and Methods

2.1. Strains and Culture Media

The non-methylating strain of *Escherichia coli* ET12567 carrying pUZ8002 was used for conjugation with *Streptomyces*, as described in Kieser et al. [40]. Additionally, 2xTY (tryptone yeast) (16 g tryptone, 5 g NaCl, 10 g yeast extract per L) with appropriate antibiotics were used to grow *Escherichia coli* ET12567 carrying pUZ8002. SFM (soya flour mannitol) [40] with 0.1 mM $MgCl_2$ was used for conjugation, as described in Kieser et al. [40]. *Streptomyces* from the CS collection strains (CSs) were grown in SFM and MA (A Medium) [41]

for sporulation. The strains were kept as spores in 50% glycerol at −20 °C. For secondary metabolite production, strains were grown in five different production media: SM10 [42], R5A [43], SM17 (composition per litre: glucose 2 g, glycerol 40 g, soluble starch 2 g, Arkasoy (soy protein) 5 g, peptone 5 g, yeast extract 5 g, NaCl 5 g, $CaCO_3$ 2 g, tap water), SM20 [44], and YEME-S (yeast extract-malt extract without sucrose) [40] containing different sources of carbon or nitrogen. *Streptomyces* strains used in this study are listed in Table S1 in Supplementary material.

2.2. Gene Selection

Genes selected for this study were carefully chosen based on the available bibliographical information showing the role of each gene in the metabolism of different *Streptomyces* species and, when available, the effect that their overexpression and/or deletion has on their secondary metabolites production (Tables 1 and 2). In the case of *rpoB*, point mutations are known to activate secondary metabolism [39]. However, this gene was selected for overexpression in an effort to improve the quantity of available RNA polymerase in the cell and, consequently, to reinforce the transcriptional machinery.

2.3. pSETxk Construction

To construct plasmid pSETxk, the *kasOp** constitutive promoter was extracted from plasmid pDR4-K* [36] by digestion with BamHI/SpeI. The released DNA fragment was blunt-ended by T4 DNA polymerase and cloned in the PstI site (blunt-ended) of plasmid pOJ260 [45]. The correct orientation of the promoter relative to the multiple cloning site in the resulting plasmid (pOJ260k) was confirmed by PCR with primers dKAS-check and rvKAS-check. Then, a 1.8kb-DNA fragment containing genes *xylE* and *neoR* was amplified by PCR from pDR4-K* with primers SmaI-NsiI-REP and MunI-REP, and cloned in the EcoRV/EcoRI sites of pOJ260k to afford plasmid pOJk-REP. Finally, a 2.1kb-DNA stretch encompassing kasOp*, xylE, and neoR was amplified from pOJk-REP with primers BglII-KasOd and MunI-REP and inserted into the BamHI/EcoRI sites of plasmid pSET152 [45] to produce pSETxk (Figure 1). (Primers are summarized in Table S2, Supplementary Material).

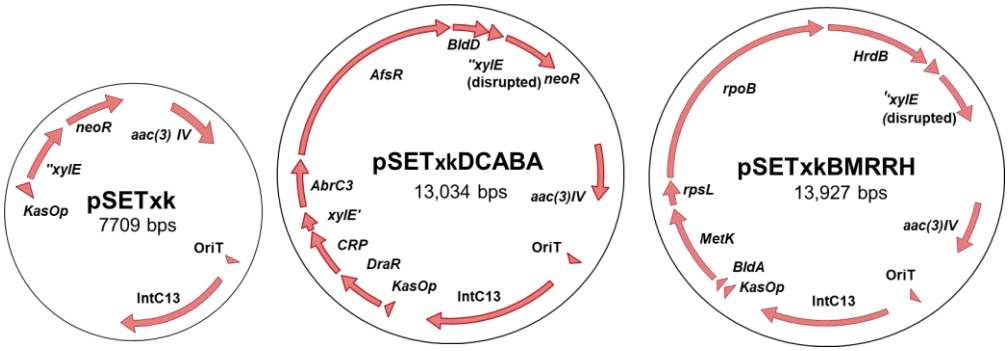

Figure 1. Schema of the constructs used in this work. From left to right, pSETxk, pSETxkDCABA, and pSETxkBMRRH. pSETxkDCABA and pSETxkBMRRH have fragmented *xylE* since an enzyme has been used that has disrupted this gene, which is not necessary for this experiment.

2.4. Construction of a Multiregulator Recombinant Plasmid

Genes were amplified from *Streptomyces coelicolor* chromosome using primers shown in Table S2 in Supplementary material. Primers were designed to amplify each gene with an individual restriction site at each side to facilitate directed and consecutive cloning of the genes. All genes were cloned in a single operon controlled by the strong constitutive promoter *kasOp*, with the promoterless gene *neoR* at the end of the operon, serving as an indication that the expression of all genes is correct when the recombinant strains were resistant to kanamycin. The amplification of each gene was designed in such a way that includes the native RBS (ribosomal binding site) and avoids the inclusion of the transcriptional terminator that would stop the expression of the artificial operon. The

EcoRV restriction site was needed to be able to clone all the genes, which truncated the *xilE* gene that was not necessary for this work.

The final constructs were designated pSETxkDCABA, which contains the genes *draR*, *CRP* (cyclic AMP receptor protein*)*, *abrC3*, *bldD* and *afsR*, and pSETxkBMRRH, which contains *bldA*, *metK* (methionine adenosyltransferase), *rpsL* (30S ribosomal protein S12), *rpoB* (RNA polymerase subunit beta), and *hrdB* (Figure 1).

2.5. Strain Construction

PSETxkDCABA, pSETxkBMRRH, and the empty vector pSETxk were introduced in wild-type strains of *Streptomyces* through conjugation, following the protocol in Keiser et al. [40]. To verify whether each strain contained all the genes cloned in each construct, the genomic DNA of each strain was extracted. PCR amplifications were performed on at least two contiguous regulators, using the free constructs as a positive control. Furthermore, resistance to apramycin and kanamycin was confirmed. The primers used for strain confirmation are listed in Table S3 Supplementary material.

2.6. Generation of Spontaneous Mutants Resistant to Rifampicin and Streptomycin

Wild-type strains were cultured on MA supplemented with 50 µg/mL streptomycin or 100 µg/mL rifampicin to obtain spontaneous single mutants [46,47]. Subsequently, they were cultured on MA with 100 µg/mL streptomycin or 200 µg/mL rifampicin to verify the acquisition of resistance. pSETxkDCABA, pSETxkBMRRH, and the empty vector pSETxk were also introduced in these strains.

2.7. Production of Secondary Metabolites in Liquid Media

Spores from each strain were used to inoculate the precultures in TSB (tryptic soy broth), using baffled flasks for proper dispersion of mycelia. In total, 50 mL of each media were inoculated from the precultures, with an initial O.D. of 0.2. Cultures were grown for 13 days at 250 rpm and 30 °C. Whole culture samples were taken after 4, 6, 8, and 13 days. The samples were extracted with three different organic solvents: ethyl acetate, acidic ethyl acetate (1% formic acid), and butanol. Samples were left to mix with the solvent for 1–2 h of shaking. The solvent phase was then separated by centrifugation and dried under vacuum (Labcono CentriVap Benchtop Vacuum Concentrator). For each set of strains and media (for example, CS014 wild-type, and recombinant strains from this parent strain in SM10), the sample with the highest dry weight was resuspended in 100 µL of methanol and the rest of the samples were resuspended in a proportional volume to the mass of the dry weight to keep a similar concentration for all samples. Then, 10 µL were injected into the UPLC and LC/MS for analysis. The amount of methanol added to the samples was normalized to the dry weight of mycelia.

2.8. Secondary Metabolites Production on Solid Media

Spores were plated on R5A agar and YEME-S agar for 5 days [48]. This modified version of YEME-S (without sucrose) was used to limit the available carbon source and it is useful for the analysis of growth and antifungal production. Then, 3.5 g of each culture was extracted with three different solvents: ethyl acetate, acidic ethyl acetate (1% formic acid), and butanol. After 1–2 h of mixing with solvents, the organic phase was collected and evaporated in the same way as described above. Subsequently, the dried extracts with the highest dry weights were resuspended in 100 µL of methanol and the rest of the samples were resuspended in a proportional volume to the mass of the dry weight to keep a similar concentration for all samples. Then, 10 µL of samples were injected into the UPLC.

2.9. Bioactivity Analysis from Solid-Media Samples

Agar diffusion bioassays against *Micrococcus luteus* (Gram-positive), *Escherichia coli* (Gram-negative), and the yeast *Candida albicans* were performed to test for the antibiotic production. TSA (agar tryptic soy both) were used for *M. luteus* and *E. coli* assays and YMA

(yeast extract 3 g; malt extract 3 g; peptone 5 g; and glucose 10 g per litre) was used for *C. albicans* assays. Two different bioassays were performed: (i) a 6 mm agar plug from each actinobacteria culture (grown as described in 2.8 section) was placed on top of the bioassay plate; and (ii) solvent-extracted samples were resuspended on methanol and 20 µL of each sample was added into a diffusion bioassay disc. The plates were then incubated at 4 °C for one hour to allow the metabolites to diffuse into the surrounding medium and finally incubated for 16 h at 30 °C (antifungal tests) or 37 °C (antibacterial tests). The diameter of the inhibition zones was measured and compared with the control sample. Each test was performed in triplicate.

2.10. Chromatographic Analysis

Samples were run on an Acquity UPLC I-Class (Waters, Mildford, MA, USA) using a BEH C18 column (1.7 µm particle size, 2.1 mm × 100 mm) and acetonitrile and water containing 0.1% of trifluoroacetic acid as mobile phase. A gradient was used from 10 to 99% of acetonitrile in 10 min and a flow rate of 0.5 mL/min. For HPLC/MS analysis, a Waters ZQ4000 system was used connected to an HPLC 2695/2795 (An Alliance chromatographic system coupled to a SunFire C18 column (3.5 µm particle size, 2.1 mm × 150 mm) and a 996 PDA detector. Acetonitrile and MQ water + formic acid 0.1% were used as the mobile phase and elution was performed with an isocratic hold with acetonitrile (10%) for 4 min followed by a linear gradient of acetonitrile (10–88%) over 30 min (0.25 mL/min). Mass analysis was performed by ESI (electrospray ionization) in the positive mode with a capillary voltage of 3 kV and cone voltage of 20 kV. The Empower 3.0 program was used to compare and analyse the chromatograms obtained from each sample.

2.11. Prediction of Secondary Metabolites Biosynthetic Gene Clusters

Web-based software antiSMASH 7.0 was used to analyse the genomic sequence of these strains to detect the putative secondary metabolites gene clusters present in their chromosomes [49,50]. It was considered that the prediction is accurate when the percentage of identity is greater than 85%.

2.12. Dereplication Assay

HRMS-based compound dereplication was performed at Medina Foundation. The in-house library and the Dictionary of Natural Products version 26:2 were used to identify already known compounds. LC-MS was performed on Agilent 1200 Rapid Resolution HPLC. Analysis was performed on a maXis Bruker qTOF mass spectrometer. The volume injected was two µL and a Zorbax SB-C8 column (2.1 × 30 mm, 3.5 µm particle size) was used for the separation. The mobile phase consisted of solvent A, 90:10 milliQ water-acetonitrile, and solvent B, milliQ water-acetonitrile, both with 13 mM ammonium formate and 0.01 TFA. Samples were eluted with a 0.3 mL/min flow rate, and the gradient used was 90% to 0% solvent A/10% to 100% solvent B in 6 min, 0% solvent A/100% solvent B in 2 min, 0% to 90% solvent A/10% to 100% solvent B in 0.1 min, and 90% solvent A/10% solvent B for 9.1 min. The maXisqTOF mass spectrometer was operated in ESI positive mode. Source conditions were 4 kV capillary voltage, end plate offset = 500 V, dry gas (N2) flow = 11 L/min; dry temperature = 200 °C, and nebulizer (N2) pressure at 2.8 bars. The retention time, together with the exact mass and the derived molecular formula, was used as the criteria to search in databases.

3. Results and Discussion

Five different pleiotropic regulators (Table 1) and five different housekeeping genes (Table 2) were selected to be overexpressed in *Streptomyces* sp. strains by the constructions pSETxkDCABA and pSETxkBMRRH, respectively (Figure 1). These regulators were selected based on bibliographic references where higher production of one or more of the known antibiotics from *Streptomyces* spp. was induced by the overexpression of these genes. We also selected pleiotropic genes that have shown that their overexpression or

point mutations in their sequence induced a higher production of secondary metabolites in *Streptomyces* spp. Previous studies have shown how the introduction or deletion of several regulators at the same time generates different synergistic actions and that global regulators interact with each other [18,22]. The introduction of a panel of genes will, therefore, generate a response that may not be generated with the introduction of these genes separately.

pSETxkDCABA and pSETxkBMRRH were introduced in twelve different strains of *Streptomyces* obtained from the CS strain collection [42] and in rifampicin and streptomycin resistant strains in order to modify their secondary metabolism. Rifampicin resistant mutants contain mutations in the *rpoB* gene that codes for RNA polymerase, and streptomycin resistance is accomplished by mutations in the *rpsL* gene that codes for the ribosomal protein S12. Both mutations are known to induce the production of secondary metabolites [51–53]. It was, therefore, expected that the introduction of pSETxkBMRRH or pSETxkDCABA in these resistant strains, which already possess a modified secondary metabolism, would have a different effect than in the wild-type strains. These strains were then grown in different media containing different carbon and nitrogen sources. Whole-culture samples were extracted with three different organic solvents and extracts were then analysed by UPLC. The chromatographic profiles of the extracts were compared with those of the wild-type strain and the control of each strain containing the empty vector, grown under the same conditions. When a different production profile was found, a dereplication analysis was conducted to discriminate between putative novel compounds from the already described ones. By applying this strategy, it was possible to detect the overproduction of some already known compounds and the activation of new ones. Then, the metabolic potential of each strain was analysed using antiSMASH to determine which secondary metabolites are predicted to be produced [54]. Thus, it is intended to determine a production optimization strategy to make it more efficient at an industrial level, as well as to identify new compounds. In the current era, both due to the growing increase in antibiotic resistance as well as the constant rediscovery of the same compounds, it is necessary to apply different approaches to discover new drugs and improve the efficiency of production of those already known [3]. The effects of these constructs on the metabolism of the different strains of *Streptomyces* spp. observed by chromatographic analysis were classified into five categories: overproduction of compounds, activation of the production, inhibition of the production, modification of the production as an effect of the insertion of the empty vector, and overproduction in spontaneous rifampicin/streptomycin resistant strains.

3.1. Modification of the Production Profile as an Effect of Insertion of the Empty Vector

In several cases, it was observed that the production of some secondary metabolites was reduced, increased, or even activated as a consequence of the insertion of pSETxk into the chromosome of the different CS strains. It has already been shown that the introduction of an empty vector in a bacterial strain can have an effect on its secondary metabolism [55]. Thus, Figure 2 shows some examples of how the insertion of the empty vector caused a metabolic change that led to the increased production of compounds, as is the case for coproporphyrins and alteramides in CS065a, coproporphyrins in CS207 (Figure 2B,D), or collismycins in CS149 (Figure 2D). Additionally, it caused a decrease in the production of different compounds, as is the case for cosmomycins in CS081a. In the rest of the analyses, the wild-type strain and the empty vector strains showed identical production profiles.

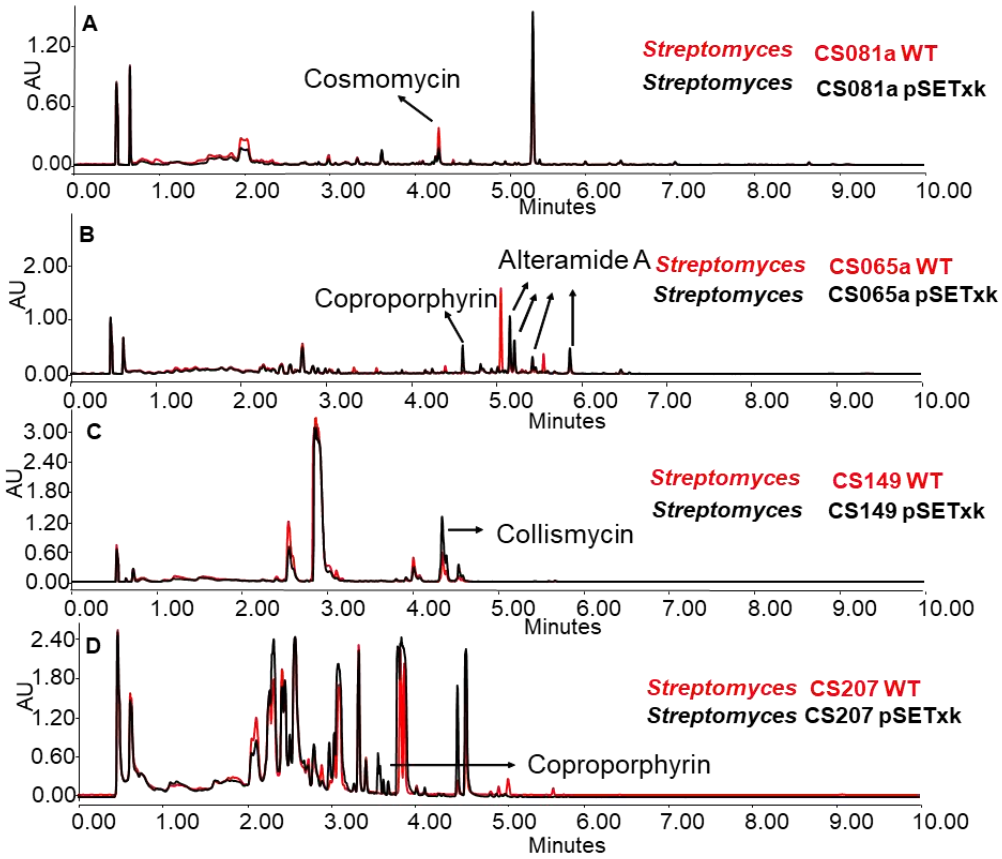

Figure 2. Comparative UPLC analysis of samples of the wild-type strain (in red colour) and the control strain carrying the empty vector (in black colour). (**A**) CS081a cultured in liquid SM10 medium at day 13 of culture and extracted with ethyl acetate. (**B**) CS065a in liquid SM20 at day 8 and extracted with ethyl acetate. (**C**) CS149 in liquid R5A at day 13 and extracted with ethyl acetate and CS207 in liquid. (**D**) CS207 in liquid SM17 at day 4 and extracted with butanol.

3.2. Overproduction of Secondary Metabolites

In most strains, we could observe an overproduction of compounds. Some of these compounds were identified by a combination of techniques. First, a bioinformatic analysis of each sequence was performed using antiSMASH, which predicted the putative biosynthetic gene clusters harboured in each chromosome. Then, HPLC and MS analysis of the extracts together with dereplication allowed us to identify compounds produced in different media by each strain. The most relevant results are shown in Table 3:

Table 3. Metabolites identified from each strain used in this work and the effect that the insertion of the described vectors had in the metabolic production of each strain. In the case that the effect was not observed in all media and extraction methods, the growth and extraction conditions where the effect was detected are specified. (↑: overproduction; ↓: inhibition; (A) activation).

Strain	Known Compounds Identified by Dereplication	Insertion of pSETxkDCABA	Insertion of pSETxkBMRRH
Streptomyces sp. CS014	Granaticin A, granaticin C, collismycin C, collismycin B, collismycin A, collismycin D, cyclo (Tyr-Pro), N-acetyltiramine, cyclo (Leu-pro), pyrosulfoxin A, cyclo (Phe-Pro), alloesaponarin II, coproporphyrins	↑ Granaticins, collismycins	↑ Granaticins, collismycins
Streptomyces sp. CS057	Cycloheximide, actiphenol, skyllamycin A, skyllamycin B, coproporphyrins	↑ Skyllamycins, actiphenol, coproporphyrins, cycloheximide	↑ Skyllamycins, actiphenol, coproporphyrins, cycloheximide

Table 3. *Cont.*

Strain	Known Compounds Identified by Dereplication	Insertion of pSETxkDCABA	Insertion of pSETxkBMRRH
Streptomyces sp. CS065a	Alteramides, chromomycin A3, chromomycin Ap, chromomycin A2, coproporphyrins	↓ Chromomycins only in SM10 media ↑ chromomycins in R5A, SM17 and SM20 (A) chromomycins in YEME-S	↑ Alteramides coproporphyrines in SM20
Streptomyces sp. CS081a	Dihydrotetrodecamycin, cosmomycin C, coproporphyrins	↑ Dihydrotetrodecamycin	↑ Cosmomycins (SM10 and SM17 extracted with ethyl acetate and ethyl acetate 1% formic acid)
Streptomyces sp. CS090a	Maltophilin, alteramides, 2-aminobenzoic acid, coproporphyrins	↑ Maltophilins (YEME-S), 2-aminobenzoic acid (SM10)	↑ Maltophilins (YEME-S and R5A), 2-aminobenzoic acid (SM10) (A) alteramides
Streptomyces sp. CS113	2,4-dihydro-2-hydroxy-1(2h)-isoquinolinone, germicidin A, cervimycin A, coproporphyrins, seitomycin, cyclo(phenylalanylprolyl), papuline, aurantimycin	-	↑ Germicidin (R5A and SM10
Streptomyces sp. CS131	Actinomycin D, I, G4, X2, coproporphyrins	↑ Actinomycins (m10, SM17, and R5A)	-
Streptomyces sp. CS147	Colibrimycins, vicenistatin, cyclo (leu-Pro), coproporphyrins, N-acetyltyramine, N-chloroacetyl tryptophan	↑ Antibiotic cyclo (leu-pro), colibrimycins, N-acetyltyramine ↑ coproporphyrins (SM17 and SM10) ↑ N-chloroacetyl tryptophan(R5A)	↑ Antibiotic cyclo (leu-pro), colibrimycins, N-acetyltyramine ↑ coproporphyrins (SM17 and SM10) ↑ N-chloroacetyl tryptophan(R5A)
Streptomyces sp. CS149	Collismycin A, B, C, D, F, coproporphyrins	Coproporphyrins (SM17 and SM10) ↑ bioactive compound against Gram-positive in R5A	Collismycins coproporphyrins ↑↑ bioactive compound against Gram-positive in R5A
Streptomyces sp. CS159	Undecylprodiogiosin, inthomycins, coproporphyrins	↑ Inthomycin (R5A)	↑ Inthomycin (R5A and SM10) ↑ undecylprodigiosin (R5A)
Streptomyces sp. CS207	3-(2-hydroxiethyl)-6-prenylindole, 3-cyanomethyl-6-prenylindole, coproporphyrins	↑ 3-cyanomethyl-6-prenylindone, 3-(2-hydroxyethyl)-6-prenylindole (R5A, ethyl acetate, ethyl acetate 1% formic acid) ↑ coproporphyrins (SM17 and R5A)	↑ 3-cyanomethyl-6-prenylindone, 3-(2-Hydroxyethyl)-6-prenylindole (R5A, ethyl acetate, ethyl acetate 1% formic acid)
Streptomyces sp. CS227	Surugamide A, 2-aminobenzoic acid, abenquine A, coproporphyrins	↑ 2-aminobenzoic acid and surugamide A	↑ 2- aminobenzoic acid and surugamide A

CS014: this strain is known to produce granaticin, a coloured antibiotic from the benzoisochromanequinone family polyketide, to which actinorhodin belongs too, and collismycins antibiotics [56]. When the recombinant plasmids constructed for this work were inserted in this strain, an overproduction was observed of both compounds in all of the tested conditions (Figure 3).

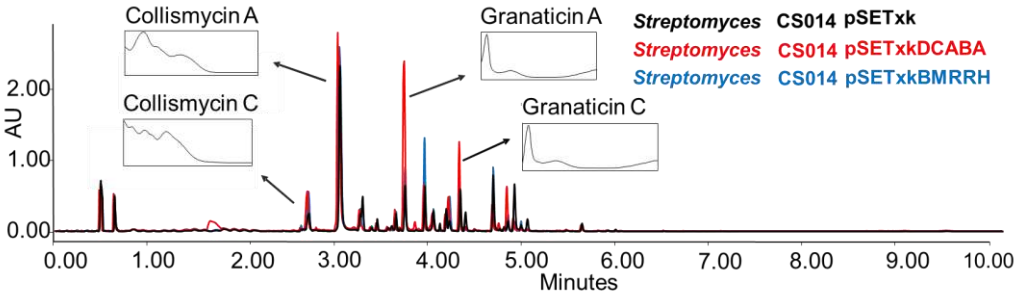

Figure 3. Comparative UPLC analysis of CS014 samples cultured in liquid SM10 medium after four days of growth and extraction with ethyl acetate. The ultraviolet-visible (UV-vis) spectra of the detected collismycin and granaticin compounds are shown.

CS057: This strain is known to produce the strong inhibitors of platelet-derived growth factor skyllamycin A and B [57], actiphenol, and cycloheximide, which both inhibit eukaryotic translation [58,59]. All three compounds have been overproduced by both recombinants in R5A, SM17, and SM10 media. Furthermore, the production of coproporphyrin, a metal chelate [60,61], was activated in SM10 and SM20 media (Figure 4).

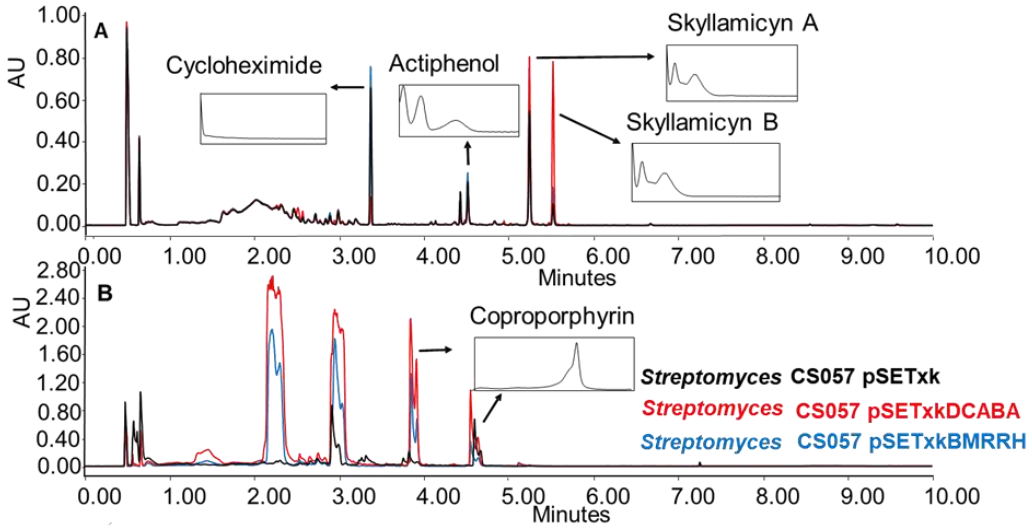

Figure 4. (**A**) Comparative UPLC analysis of CS057 samples cultured in liquid R5A medium at day 8 of culture and extracted with ethyl acetate. (**B**) Comparative UPLC analysis of samples of CS057 cultured in liquid SM10 medium at day 8 of culture and extracted with ethyl acetate with 1% formic acid. UV-vis spectra of the detected compounds are shown.

CS113: When carrying the construct pSETxkBMRRH, this strain overproduced germicidin, a germination-inhibitor compound A [62,63] when cultured in R5A and SM10 media and extracted with any of the solvents tested (Figure 5).

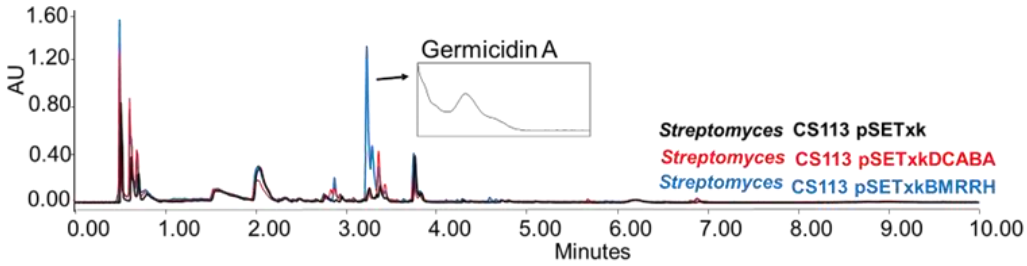

Figure 5. Comparative UPLC analysis of CS113 samples cultured in liquid R5A medium at day 8 of culture and extracted with ethyl acetate. UV-Vis spectrum of germicidin A shown.

CS147: In all of the media tested, both mutants overproduced the antibiotic Cyclo (leu-pro), colibrimycins (a hybrid polyketide synthase-nonribosomal peptide synthetase only detected when cultures were extracted with ethyl acetate containing formic acid or butanol) and N-acetyltyramine (with antimicrobial properties,) (Figure 6) [64–67]. Additionally, in SM17 and SM10, the overproduction of coproporphyrins could be observed by both recombinants. In R5A, N-chloroacetyl tryptophan was overproduced by both recombinants when cultures were extracted with butanol.

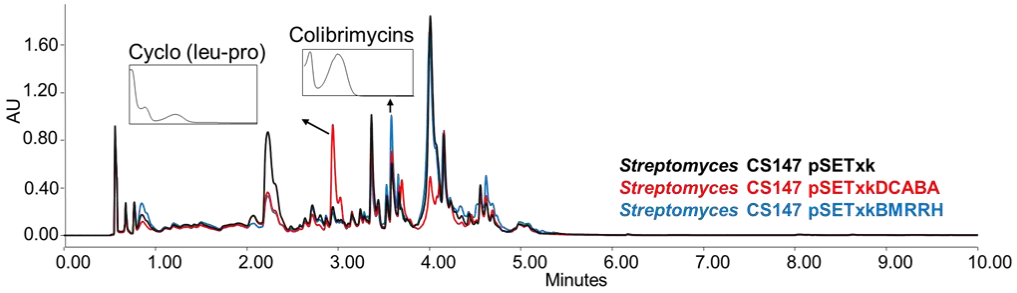

Figure 6. Comparative UPLC analysis of CS147 samples cultured in liquid R5A medium at day 4 of culture and extracted with ethyl acetate with 1% formic acid. UV-vis spectrum of cyclo (Leu-Pro) and colibrimycins are shown.

CS149: CS149 bearing pSETxkBMRRH showed an overproduction of collismycins in all media tested. Coproporphyrins were also overproduced in SM17 and SM10 media by both recombinants. Bioassay of the extracts obtained from the culture on agar R5A (with the three solvents, ethyl acetate, ethyl acetate with 1% formic acid, and butanol) of the recombinants of this strain showed differential bioactivity against *M. luteus.* The empty vector control generated a growth inhibition zone of 11 mm of diameter in the bioassay, pSETxkDCABA a halo of 20 mm of diameter, and pSETxkBMRRH a halo of 24 mm of diameter (Figure 7). This activity may be due to the distinctive production of collismycin, although the height of the peaks from the empty vector control and the strain carrying pSETxkDCABA is quite similar while the size of the inhibition halo is almost double the one observed in the empty vector control, which could mean that the bioactivity observed is caused by a different compound not detected by UPLC.

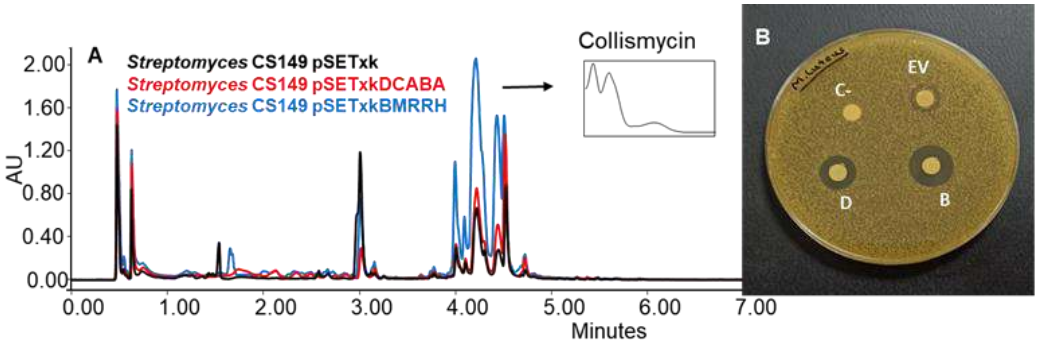

Figure 7. (**A**) Comparative UPLC analysis of C149 samples cultured on agar R5A medium and extracted with butanol. UV-vis spectrum of collismycinis shown. (**B**) Bioassay of the samples extracted with butanol from solid R5A and resuspended in methanol against *M. luteus.* In total, 20 µL methanol was used as negative control (C-). EV indicates where the sample from CS149 pSETxk was spotted; D indicates the sample of CS149 pSETxkDCABA; and B the sample of CS149 pSETxkBMRRH.

All of the results shown in this work are a summary of an exhaustive screening of metabolite production on solid and in liquid media and the application of various methods of extraction, with the aim of analysing the production of secondary metabolites. The metabolic profiles of the different recombinant strains compared to the controls (wild-type and the strain containing the empty vector), revealed a general increase in the production of compounds, many of which could be identified by dereplication. The data corresponding to the identification of compounds whose production was activated/overproduced in this work are shown in Supplementary data. In all strains, the increased production of compounds was shown by at least one of the genetic constructs introduced. However, the increase was not equally efficient in all strains or for all compounds they produce; it was influenced by the test conditions (culture medium and extraction method). For each strain and compound, it is necessary to determine the ideal production conditions to observe the effect of the regulators inserted with greater efficiency.

3.3. Activation of Secondary Metabolites

The strains bearing pSETxkDCABA and pSETxkBMRRH showed, on some occasions, the activation of different metabolic pathways. Specifically, the activation of the synthesis of alteramides was observed for the CS090a pSETxkBMRRH strain cultured in R5A, while the strain containing the empty plasmid did not produce this compound or did in undetectable levels. It also overproduced maltophilins (Figure 8A). Similarly, this effect was observed in the CS065a strain where both recombinants overproduced alteramides and the production of chromomycins got activated when cultured in YEME-S medium. Extracts from these cultures showed a growth inhibition against M. luteus when tested in a bioassay. pSETxkDCABA produced an 11 mm diameter halo and pSETxkBMRRH produced a 20 mm halo. The negative control or the strain containing the empty vector did not produce any growth inhibition (Figure 8B). Presumably, this bioactivity is due to chromomycin production, since lower production was observed in CS065a pSETxkDCABA than CS065a pSETxkBMRRH, which correlates with the halo size observed from both strains (Figure 8B,C).

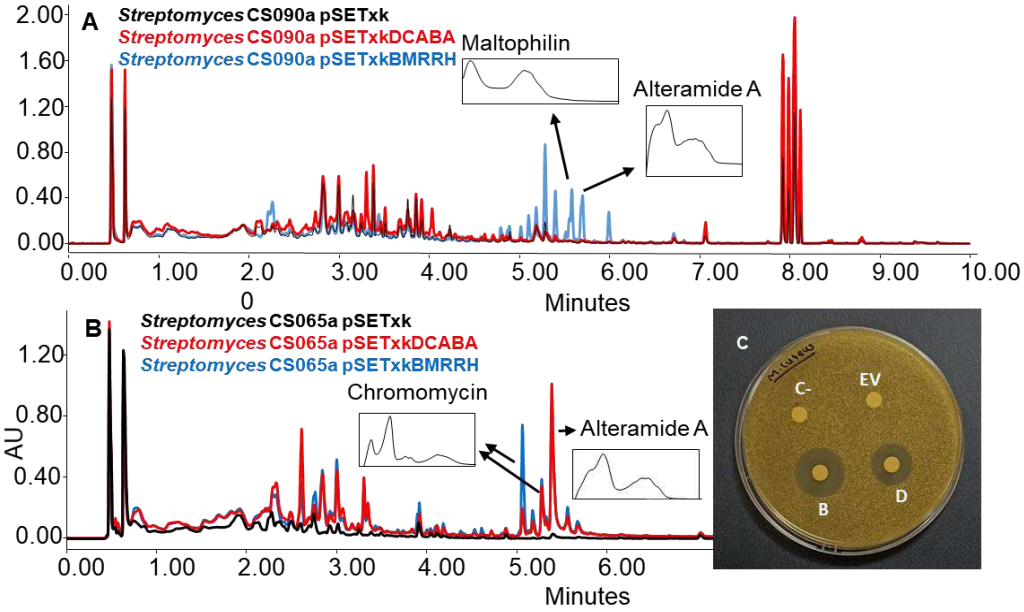

Figure 8. (**A**) Comparative UPLC analysis of samples of CS090a cultured on agar R5A medium and extracted with ethyl acetate with 1% formic acid. (**B**) UPLC analysis of samples of CS065a culture on agar YEME-S medium and extracted with ethyl acetate with 1% formic acid. UV-vis spectra of alteramide and chromomycin are shown. (**C**) Bioassay plate against M. luteus of the samples extracted with ethyl acetate with 1% formic acid and resuspended in methanol. In total, 20 µL of methanol was used as negative control (C-). EV indicates where the sample of the culture from CS65a pSETxk was assayed; D indicates where the sample from CS065a pSETxkDCABA was spotted. B shows where the sample from CS065a pSETxkBMRRH was assayed.

The de novo biosynthesis of alteramides and chromomycins by these mutants in YEME-S manifests the great potential of this genetic approach to awaken silent biosynthetic gene clusters that govern the production of bioactive compounds which may be inactive in tested conditions. An interesting observation is that the same strains grown in R5A liquid media did not show the same production as in R5A solid media, stating the importance of performing a screening with different settings conditions to determine the best production conditions for each strain and compound.

In most of the cases shown, some of the overproduced or activated metabolites were identified. Compounds that remained unidentified were either produced in too small amounts to be able to obtain a proper identification or they are not present in the library used for the dereplication. Further optimization of their production would be needed, and

isolation of the compounds followed by structural elucidation would be needed to know their structure and be able to test their bioactivity.

3.4. Inhibition of the Secondary Metabolite Production

Global regulators, also known as pleiotropic regulators, up-regulate and down-regulate the primary and secondary metabolism of bacteria [21]. In some cases, these changes result in activation or overproduction, as described in the previous sections. In other cases, they can repress or down-regulate the expression of genes that result in the inhibition of the production of other compounds [26]. From all strains analysed, we only observed one case of inhibition which was in Streptomyces CS065a bearing pSETxkDCABA and grown in SM10 media (Figure 9).

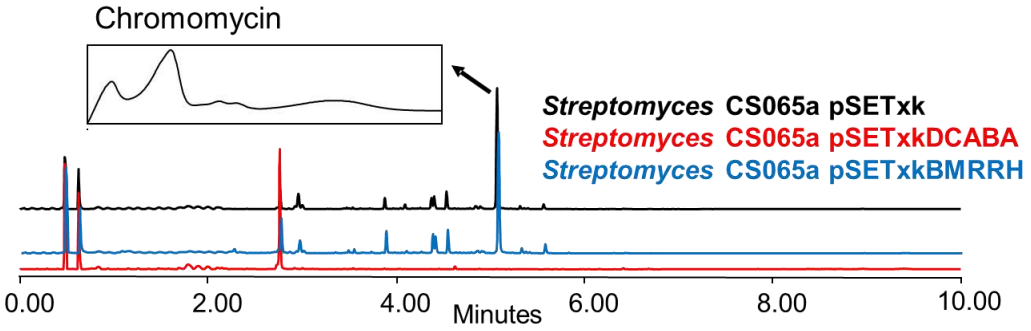

Figure 9. Comparative UPLC analysis of samples of CS065a cultured in liquid SM10 medium at day 4 of culture and extracted with ethyl acetate. UV-vis spectrum of chromomycin is shown.

The inhibition of chromomycin biosynthesis might be a consequence of a deregulation effect on metabolism that, in this case, represses the biosynthesis pathway of this compound under very specific conditions. This result is exceptional since the genetic constructs used in the present study in most cases showed an increase in the production of compounds and not their inhibition. However, the alteration of metabolism can result in activation, overexpression, or inhibition depending on the strain or media. These effects can also be appreciated to a greater or lesser extent depending on the organic solvent used since compounds are extracted differently using different solvents, hence showing the importance of testing each strain under different conditions to obtain the desirable effect. The inhibition of chromomycins was only observed in SM10 medium when the Streptomyces CS065a contained the construct pSETxkDCABA; in the rest of the cases, the strain is capable of producing them and their production is considerably increased (Figure 8).

3.5. Effect of Spontaneous Rifampicin and Streptomycin Resistant Mutations on Secondary Metabolism

pSETxkDCABA and pSETxkBMRRH were also introduced into spontaneous rifampicin (R.R.) and streptomycin (R.S.) resistant strains that were obtained as described in the Materials and Methods section. Resistance to rifampicin and streptomycin has been shown to induce changes in the metabolism by improving the level of production of bioactive compounds and activating new metabolite synthesis [53]. Rifampicin resistant mutant strains contain mutations in their RNA polymerase rpoB gene while streptomycin resistant mutants have mutations in the S12 ribosomal protein rpsL [51–53]. Strains carrying pSETxkBMRRH, apart from their mutated rpsL and rpoB genes, contain the native genes from S. coelicolor. However, the mutations were not complemented, as the strains remained resistant to rifampicin or streptomycin.

The generated mutants showed an incremented metabolic profile compared to the wild-type strain. As shown in Figure 10 as an example, the generation of spontaneous resistance increased the production of chromomycins, Cyclo (leu-pro), N-chloroacetyl tryptophan, colibrimycins, vicenistatin, and other compounds that could not be identified. As in previous results, this increase is dependent on the culture media and solvents employed, thus varying the metabolic profile. Moreover, the introduction of the constructs contributes

substantially to a further increase in production, again, depending on the media and solvent used in the extraction.

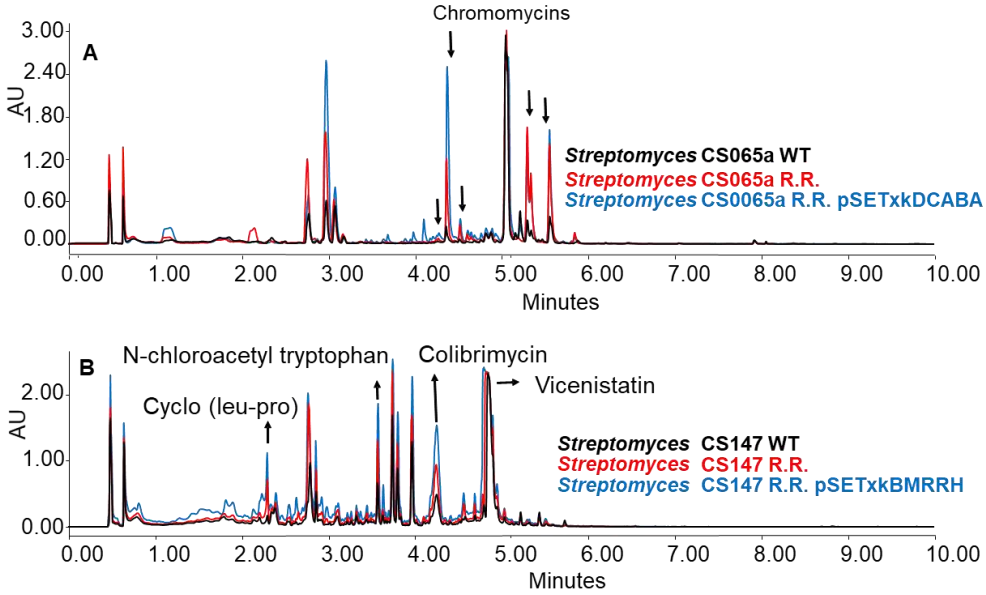

Figure 10. (**A**) Comparative UPLC analysis of samples from CS065a WT, CS065a R.R., and CS065a R.R. containing pSETxkDCABA cultured in liquid R5A medium at day 4 of culture and extracted with ethyl acetate. (**B**) Comparative UPLC analysis of samples from CS147 WT, CS147 R.R., and CS0147 R.R. containing pSETxkDCABA cultured in R5A medium at day 4 of culture and extracted with butanol.

When CS065a R.R was cultured in R5A, chromomycins were overproduced. This overproduction was further increased by the insertion of any of the constructions, especially CS065a R.R. pSETxkDCABA (Figure 10A). Similarly, when CS147 was cultured in R5A, N-chloroacetyl tryptophan, colibrimycin, and cyclo (pro-leu) were overproduced. A slight improvement in vicenistatin production by CS147 R.R. pSETxkBMRRH can be seen (Figure 10B). However, the recombinant carrying pSETxkDCABA does not show a substantial production increase.

Furthermore, CS147 streptomycin resistant mutants have a generally increased production level compared to the wild-type strain. However, when both genetic constructs were introduced into the CS147 R.S. strain, the production was not increased under the conditions tested, although the CS147 strains containing both constructs had a better general production of compounds than the wild strain (Figure 10). This fact makes us consider that this regulator strategy can be much more efficient than the acquisition of spontaneous resistance, adding that the genes responsible for such an effect are known when introducing the constructs described in this work, while it is uncertain where spontaneous point mutations are located.

This attempt to improve secondary metabolism highlights the possibility of combining the insertion of global regulators and housekeeping genes with other classical techniques to improve compound production. This does not only imply greater profitability at the industrial level, but it is also an interesting tool that can be used in research, as many cryptic biosynthetic pathways remain unknown due to difficulties in activating them or because they are produced at such low levels that it is very difficult to detect them. The activation of metabolic pathways by these techniques or the increase in the production of a compound that is already intrinsically produced by the wild-type strain offers an advantage that can lead to improving the research and discovery of new natural products. Therefore, the simultaneous use of different techniques can be an efficient strategy to consider. The main advantage offered by the constructs described in this work is that their insertion into the chromosome is highly efficient, since the use of pSETxk, an integrative plasmid, makes it relatively easy to obtain recombinants with the increased production of secondary

metabolites. On the other hand, ribosomal engineering is also another classic strategy that is easy to use to increase production [51–53]. The combination of these two approaches is just one example of the different technologies with which the use of global regulatory elements and housekeeping genes can be combined. In the case of rifampicin resistant mutants, results of increased production were obtained once the pSETxkDCABA or pSETxkBMRRH constructs were introduced. However, the insertion of the corresponding constructs in the streptomycin resistant mutants did not improve production under the tested conditions.

CRP, DraR, AbrC3, BldD, and sigma factor HrdB bind to promoters of structural genes or pathway specific regulators of different secondary metabolite biosynthetic clusters. These promoters must contain the corresponding consensus sequences so that these regulators can bind the DNA [25,31,32,68,69]. In the case of AfsR, MetK, BldA, RpoB, and RpsL, they induce the overproduction of compounds but in an indirect way. AfsK activates itself by autophosphorylating after binding to S-adenosyl-L-methyonine (SAM). Then, it phosphorylates AfsR which activates the transcription of afsS [70]. The mechanism of action of protein AfsS is not known, but it interacts with pathway specific regulators of secondary metabolites [22]. An overexpression in MetK results in high levels of S-adenosyl-L-methyonine, which is a methyl donor and has been known to directly activate transcriptional factors of antibiotic production [33]. The bldA gene is necessary for the translation of genes that contain the rare codon TTA encoding for leucine. Therefore, it will affect only genes containing the TTA codon [39]. Point mutations in RpoB are known to improve secondary metabolites production by increasing the affinity of the RNA polymerase promoter, while in RpsL, however, protein synthesis is induced in the stationary growth phase, therefore improving the production of compounds [71]. The overexpression of wild-type rpsL also showed the activation of compounds in *S. clavuligerus* [38].

Sequence analysis software antiSMASH includes in its seventh version a prediction tool of transcription factors binding sites (TFBS) with which it was possible to know which gene clusters had a binding site for the regulators used in this work. Table S4 shows the TFBS predicted with strong confidence. Only clusters shown in Table 4 had a predicted TFBS for the regulators used in this work and showed a modified production level. As observed in Table S5, many clusters in all strains contain a TTA codon. However, only a few detected a modification in their production levels when introducing pSETxkBMRRH (Table 4). Interestingly, the holomycin cluster predicted in CS014, CS131, CS147, and CS149 are predicted to possess a TTA codon, but we were not able to detect the compound in any of these strains. Moreover, CS065a has a gene cluster with 100% similarity to the maltophilin biosynthetic gene cluster and does possess a TTA codon, but maltophilin was not detected in strain, but in strain CS090a, which possesses the same gene cluster also with a TTA codon; maltophilin was detected under the same conditions. The same situation was found in CS113 and CS159, but in the case of CS159, the cluster also has a TFBS for AbrC3. However, undecylprodigiosin is overproduced when carrying pSETxkBMRRH (which contains bldA) but not with pSETxkDCABA (which contains abrC3). Regulators interact with each other, making it impossible to predict the effect that these regulators will have on a particular strain. For example, glnR expression, which controls nitrogen metabolism, and other genes from nitrogen metabolism are controlled by PhoP, which controls phosphate metabolism and they both interact with AfsR, AfsQ1 (response regulator), or DasR (chitin degradation) [21–24]. As proven by the results of this work, the same regulators have a different effect in the same biosynthetic cluster, which is most probably due to the different regulatory cascades that each strain possesses. Therefore, the prediction of the effect that these plasmids would have on a particular strain is limited.

Table 4. Clusters in each strain that have a predicted TFBS and had their product levels modified when inserting pSETxkDCABA and/or pSETxkBMRRH.

Strain	Gene Cluster Number	Gene Cluster Type and MiBiG Identification	TFBS
CS014	1.6	NRPS/PKS-I (Collismycin A 77%)	*bldA*
CS057	1.2	NRPS-like/lanthipeptide- IV/transAT-PKS (Cycloheximide 94%)	*bldA*
	1.20	Ectoine/butyrolactone/ladderane/arylpolyene/NRPS/PKS-I (Skyllamycins 97%)	*bldA*
CS065	1.23	PKS-II/oligosaccharide (Chromomycin 100%)	*bldA*
CS081a	1.4	PKS-II/oligosaccharide/PKS-like (Cosmomycin D 97%)	*bldA*
CS090a	1.21	NRPS/PKS-I (Maltophilin 100%)	*bldA*
CS113	1.22	PKS-II/PKS-like/oligosaccharide (Cervimycin 90%)	AbrC3
	1.26	Butyrolactone/PKS-III (Germicidin 100%)	AfsR
			BldD
			AbrC3
			bldA
CS131	1.6	NRPS-like/other (Actinomycin 89%)	AbrC3
			bldA
CS147	1.16	Ladderane/NRPS (Colibrimycin 75%)	*bldA*
	1.17	PKS-I/RRE-element containing cluster (Vicenstatin 100%)	*bldA*
CS149	1.21	NRPS/PKS-I (Collismycin A 70%)	*bldA*
CS159	1.5	Trans-AT PKS/NRPS/NRPS-like (Inthomycin B 100%)	*bldA*
	1.10	NRP-metallophore/NRPS/PKS-I/NRPS-like/prodigiosin (Undecylprodigiosin 100%)	*bldA*

4. Conclusions

In the era of genetic manipulation, where -omics approaches are constantly applied, there is a bottleneck in terms of the discovery of new compounds that can serve as therapeutic alternatives or new treatments. The use of integrative recombinant plasmids bearing global regulators or housekeeping genes is a relatively simple strategy that allows the improvement of bioactive compound production which can also serve as a strategy for the activation of silenced metabolic pathways. It has been verified at an extensive experimental level that, by introducing pSETxkDCABA and pSETxkBMRRH into different wild-type strains from the CS collection and combining these constructs with ribosomal engineering, the biosynthesis of metabolites is enhanced or activated. Depending on the different sources of nutrients, the improvement in the production of compounds is more noticeable. Likewise, the combination of this strategy with others such as ribosomal engineering is proposed as an alternative to incorporate other technologies. It is important to take into account that, although there has been an increase in the production of metabolites at a general level, its increase is dependent on the strain, the culture, and extraction conditions, so the use of these constructs requires performing a screening of media and culture conditions to optimize the production of the compound of interest. Even though it is possible to predict binding sites for the regulators used in this work, as the results showed, it is not guaranteed that a specific cluster is going to be affected by the insertion of these plasmids due to the different regulation levels that each strain possesses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11061585/s1, Table S1: Streptomyces strains used in this study; Table S2: Primers used in this study. Table S3: Primers used for strain confirmation. Figure S1: Comparative UPLC analysis of CS065a samples Figure S2: Comparative UPLC analysis of CS081a samples. Figure S3: Comparative UPLC analysis of CS090a samples. Figure S4: Comparative UPLC analysis of CS131 samples. Figure S5: Comparative UPLC analysis of CS159 samples. Figure S6: Comparative UPLC analysis of C207 samples. Figure S7: Comparative UPLC analysis of C227 samples. LC-MS dereplication: Data corresponding to the identification of compounds whose production was activated in this work. Table S4: Transcription Factor Binding Sites (TFBS) involved in this work find with strong confidence in the chromosome of each strain using antiSmash 7.0. Table S5: TTA codons find in the chromosome of each strain using antiSmash 7. References [72–80] are cited in the supplementary materials.

Author Contributions: Conceptualization, A.C. and C.O.; methodology, A.C., M.G.M., R.G.-S. and L.C.; software, A.C. and L.C.; validation, A.C., M.G.M., L.C. and C.O.; formal analysis. A.C., M.G.M., L.C. and C.O.; investigation, A.C., M.G.M., L.C., C.M. and C.O.; resources, C.M., J.A.S. and C.O.; data curation, A.C. and L.C.; writing—original draft preparation, A.C. and L.C.; writing—review and editing, A.C., L.C., C.M., J.A.S., C.O. and M.G.M.; visualization, A.C., M.G.M., L.C. and C.O.; supervision, A.C., J.A.S. and C.O.; project administration, C.M., J.A.S. and C.O.; funding acquisition, C.M., J.A.S. and C.O. All authors have read and agreed to the published version of the manuscript.

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Supplementary data

Table S1: *Streptomyces* strains used in this study

Strain	Construct	Reference
Streptomyces sp. CS014	WT	[14,15,57]
Streptomyces sp. CS057	WT	[14,15,57]
Streptomyces sp. CS065a	WT	[14,15,57]
Streptomyces sp. CS081a	WT	[14,15,57]
Streptomyces sp. CS090a	WT	[14,15,57]
Streptomyces sp. CS113	WT	[14,15,57]
Streptomyces sp. CS131	WT	[14,15,57]
Streptomyces sp. CS147	WT	[14,15,57]
Streptomyces sp. CS149	WT	[14,15,57]
Streptomyces sp. CS159	WT	[14,15,57]
Streptomyces sp. CS207	WT	[14,15,57]
Streptomyces sp. CS227	WT	[14,15,57]
Streptomyces sp. CS014 pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS057 pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS065a pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS081a pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS090a pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS113 pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS131 pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS147 pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS149 pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS159 pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work

Streptomyces sp. CS207 pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS227 pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS014 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS057 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS065a pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS081a pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS090a pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS113 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS131 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS147 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS149 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS159 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS147 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS149 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS159 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS207 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS227 pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS14 streptomycin resistant (S.R.)	WT	This work
Streptomyces sp. CS14 streptomycin resistant (S.R.) pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS14 streptomycin resistant (S.R.) pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS065a rifampicin resistant (R.R.)	WT	This work

Streptomyces sp. CS065 rifampicin resistant (R.R.) pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS065a rifampicin resistant (R.R.) pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS065a streptomycin resistant (S.R.)	WT	This work
Streptomyces sp. CS065a streptomycin resistant (S.R.) pSETxkDCABA	pSETxkDCABA: *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS065a streptomycin resistant (S.R.) pSETxkBMRRH	pSETxkBMRRH: *bldA, metK, rpsL, rpoB, hrdB*	This work
Streptomyces sp. CS147 rifampicin resistant (R.R.)	WT	This work
Streptomyces sp. CS147 rifampicin resistant (R.R.) pSETxkDCABA	pSETxkDCABA. *draR, CRP, abrC3, bldD, afsR*	This work
Streptomyces sp. CS147 rifampicin resistant (R.R.) pSETxkBMRRH	pSETxkBMRRH. *bldA, metK, rpsL, rpoB, hrdB*	This work

Table S2: Primers for the generation of the plasmids used in this study.

Name	**Sequence 5-->3**
d-KAS-check	GTGTTGTAAAGTCGTGGCCAGG
rvKAS-check	TGTGGAATTGTGAGCGGATA
SmaI-NsiI-REP	ATATCCCGGGATGCATTGAGTTGAAGAGGTGACGTCA
MunI-REP	ATATCAATTGACGAATTCGAGCTCGGTACC
BglII-KasOd	GTGTAGATCTTGTAAAACGACGGCCAGTG
AbrC3 Fw Ndei	AATACATATGACCTGTCGCACGGACGGTTC
AbrC3 Rv ecorv bcui	AATGATATCATACTAGTGGTGCTTCCGAGGCATGG
AfsR Fw BcuI	ATATACTAGTCTGACGTGGTTGCTCAGGATG
AfsR Rv AanI	ATATTTATAATCACCGCGCCACACTGC
BldA Fw BamHI	ATATGGATCCTGGAACCTTCACCGATGGT
BldA Rv NotI	ATATGCGGCCGCGCCCGGATGGTGGAATG
BldD Fw BcuI AanI	AATTACTAGTATTTATAACCGCGTCGACACCTTGTCC
BldD Rv EcoRV	AATTGATATCTCAGAGCTCGTCGTGGGA
CRP-FW NotI	AATTGCGGCCGCAGCCGGTCGACAAGGAGAG
CRP-Rv NsiI	AATTATGCATTCAGCGGGAGCGCTTGG
DraR Fw BamHI	ATATGGATCCCGATCTTGCCCCGGGCGTTG
DraR Rv XK NotI	AATTGCGGCCGCCTCTTACCCGGAAGGCCCTC
HrdB Fw EcorV	ATATGATATCGCCGGAAGCCGTTTTTCAAC
HrdB Rv BcuI	ATATACTAGTGTACCGCCGGTCCGTACG

MetK Fw NotI	ATATGCGGCCGCCGTCCACCAATGATCAGCCA
Metk Rv NsiI	ATATATGCATGTGCGCCCCGGATCCTTA
RpoB Fw NdeI	AATACATATGGCGCGCGTAGTGAGTCCG
RpoB Rv EcoRV BcuI	ATATGATATCATACTAGTTGACTCCCGTCAGACCTCTT
RpsL Fw NsiI	AATTATGCATCTTCACCATTCGGCACACAGAAACC
RpsL Rv NdeI	AATACATATGTTACTTCTCCTTCTTGGCGCCGTA

Table S3: Primers used for strain confirmation. DraR Fw and Crp Rv, afsR Fw and bldD Rv and bldD Fw and Neo Rv were used to confirm the presence of pSETXK-DCABA and bldA Fw and metK Rv, rpoB Rv and rpsL Fw and rpoB Fw and hrdB Rv were used to confirm the presence of pSET BMRRH.

Construct checked	**Genes amplified**	**Primers**	**Sequence**
pSETxkDCABA	*draR, CRP*	**draR Fw**	ATATGGATCCCGATCTTGCCCCGGGCGTTG
		Crp Rw	AATTATGCATTCAGCGGGAGCGCTTGG
	afsR, bldD	**afsR Fw**	GAGCACCTTGCCGTTGTAGT
		bldD Rw	GACACCTCGCTGCTCGAA
	bldD, abRC3	**bldD Fw**	AATTACTAGTATTTATAACCGCGTCGACACCTTGTCC
		Neo Rw	ATTAAGAGCTCGAACCCCAGAGTCCCGCTCAG
pSETxkBMRRH	*bldA, metK*	**bldA Fw**	ATATGGATCCTGGAACCTTCACCGATGGT
		metK Rw	ATATGCGGCCGCCGTCCACCAATGATCAGCCA
	rpsL, rpoB	**rpsL Fw**	GCAGGACAAGGTCGAGAAGA
		rpoB Rw	CTGGTGCCGAAGAACTCCT

	rpoB, hrdB	**rpoB Fw** **hrdB Rw**	GCAGGACAAGGTCGAGAAGA GCAGGACAAGGTCGAGAAGA

Additional results obtained

CS065a

An overproduction of chromomycins was detected in CS065a, both strains in different media, except for the strain containing pSETxkDCABA grown in SM10 where chromomycin production was inhibited, which will be discussed in the Inhibition section. Chromomycins have anti-cancer activity and are widely used as DNA staining since they bind to GC rich DNA [1]. In the culture of strain bearing pSETxkBMRRH grown in SM20 media, the antifungal compounds alteramides [2], and coproporphyrins were also overproduced (Figure S1, Supplementary material).

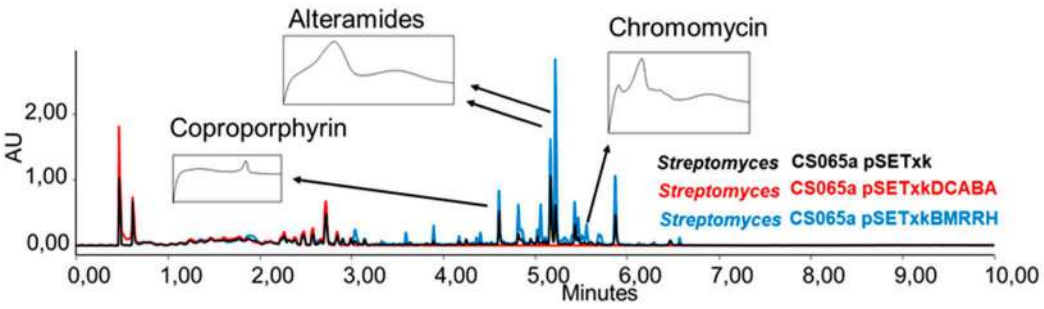


Figure S1. Comparative UPLC analysis of CS065a samples cultured in liquid SM20 medium at day 8 of culture and extracted with ethyl acetate: overproduction of chromomycin, alteramides, and coproporphyrin by pSETxkBMRRH construct can be observed. UV-Vis spectra of these compounds are shown.

CS081a:

CS081a: Cultivation of CS081a carrying pSETxkBMRRH construct in SM10 and SM17 media and extracted with ethyl acetate or acetyl acetate with 1% formic acid, showed an increased production of antitumor compound cosmomycin D [3]. Additionally, the recombinant strain bearing pSETxkDCABA also showed an increased production of dihydrotetrodecamycin (Figure S2, Supplementary material).

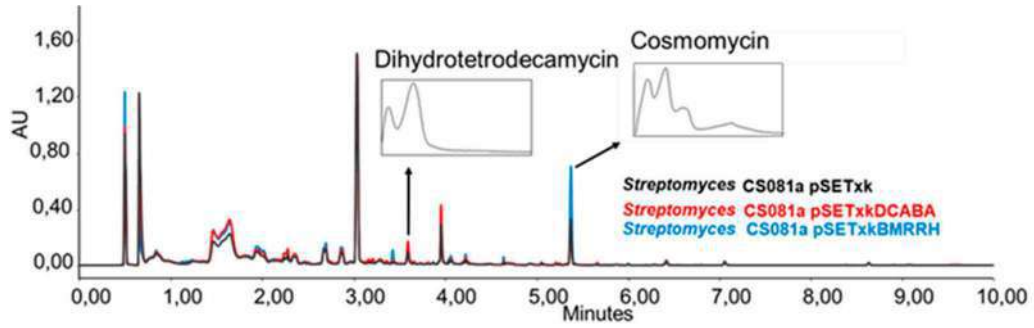


Figure S2 Comparative UPLC analysis of CS081a samples cultured in liquid SM10 medium on day 8 of culture and extracted with ethyl acetate with 1% formic acid: overproduction of cosmomycin by CS081a pSETxkBMRRH can be observed. Both recombinants overproduce dihydrotetrodecamycin. UV-Vis spectra of these compounds are shown.

CS090a:

CS090a: CS090a carrying pSETxkDCABA or pSETxkBMRRH cultured on agar YEME-S and extracted with any of the tested solvents showed an overproduction of maltophilins antibiotics [4] and alteramides. *Streptomyces* CS090a pSETxkBMRRH cultured on agar R5A (discussed in the Activation section) overproduced maltophilins and activated the biosynthesis of alteramides. Furthermore, when grown in SM10 media, both mutants overproduced the bioactive compound 2-aminobenzoic acid also known as anthranilic acid or vitamin L1 [5-7](Figure S3, Supplementary material).

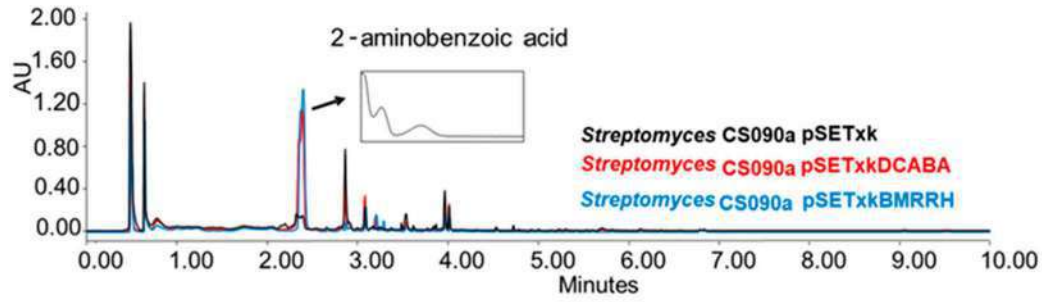


Figure S3. Comparative UPLC analysis of CS090a samples cultured in liquid SM10 medium at day 4 of cultivation and extracted with ethyl acetate with 1% formic acid: overproduction of 2-aminobenzoic acid by both mutants can be observed. UV-Vis spectrum of this compound is shown.

CS131:

CS131: An increased production of antibiotic actinomycin D was detected in recombinants carrying any of the constructs and grown in SM17, R5A, and SM10 media [8,9]. Furthermore, it was observed, especially in R5A, an activation of the production of different types of actinomycins (actinomycin I and actinomycin G4) that the wild strain does not produce or does it in undetectable levels under the conditions and with the methodology tested (Figure S4, Supplementary material).

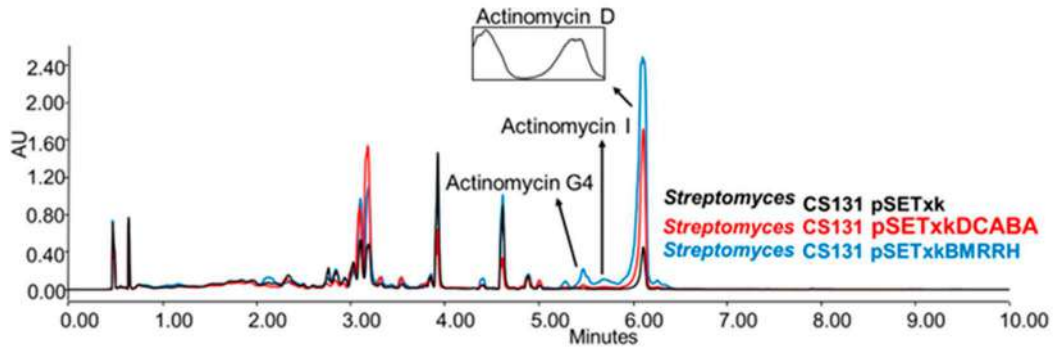


Figure S4: Comparative UPLC analysis of CS131 samples cultured in liquid R5A medium at day 13 of culture and extracted with ethyl acetate with 1% formic acid: overproduction of actinomycins by both mutants can be observed. UV-Vis spectrum of these compounds is shown.

CS159:

CS159: Both recombinants of this strain overproduced herbicide and antifungal compound inthomycin when cultured in R5A [10]. The strain carrying pSETxkBMRRH also overproduced this compound in SM10 and SM17. In addition, the overproduction of the antibiotic undecylprodigiosin can be observed in the R5A culture (Figure S5, Supplementary material) [11].

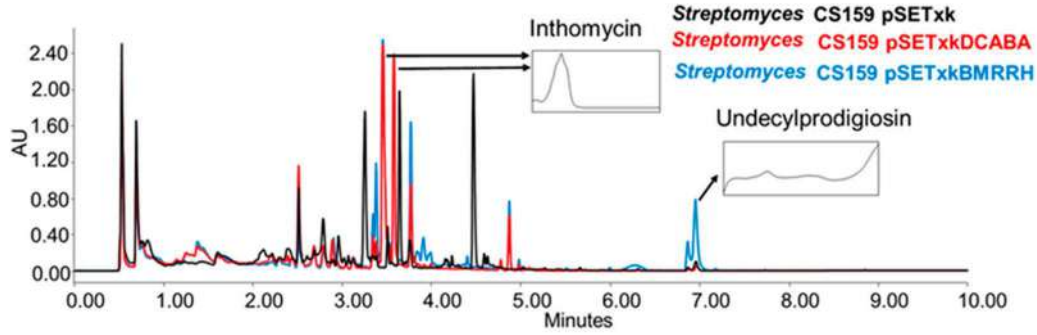


Figure S5. Comparative UPLC analysis of CS159 samples cultured in liquid R5A medium at day 13 of culture and extracted with ethyl acetate: overproduction of inthomycin by both clones and undecylprodigiosin by pSETxkBMRRH construct can be observed. UV-Vis spectra of these compounds are shown.

CS207:

CS207: Both recombinants of this strain overproduced derivative forms of bioactive compoun prenylindol, such as 3-cyanomethyl-6-prenylindone and 3-(2-Hydroxyethyl)-6-prenylindole when cultured in R5A(extracted with both ethyl acetate or ethyl acetate with 1% formic acid). In addition, in SM17 and R5A the pSETxkDCABA clone overproduces coproporphyrins (Figure S6, Supplementary material) [60].

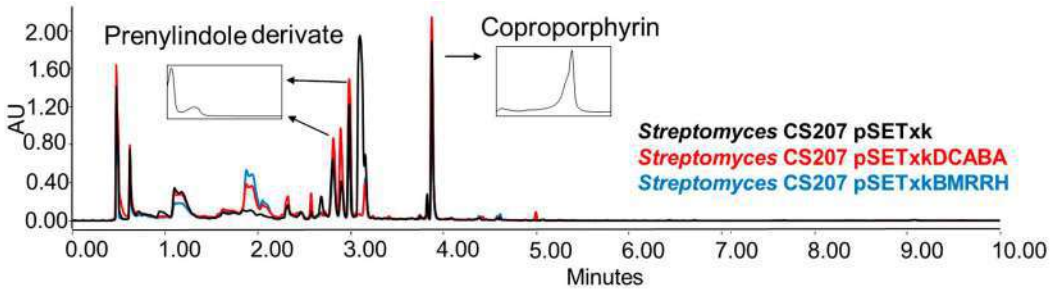


Figure S6. Comparative UPLC analysis of C207 samples cultured in liquid R5A medium and extracted with ethyl acetate with 1% formic acid: overproduction of several prenylindole derivatives by both clones could be observed. Moreover, increased production of coproporphyrins by pSETxkDCABA carrying strain can be observed. UV-Vis spectra of these compounds are shown.

CS227:

CS227: Overproduced 2- aminobenzoic acid and surugamide A in all the media tested by both recombinants (Figure S7, supplementary material)

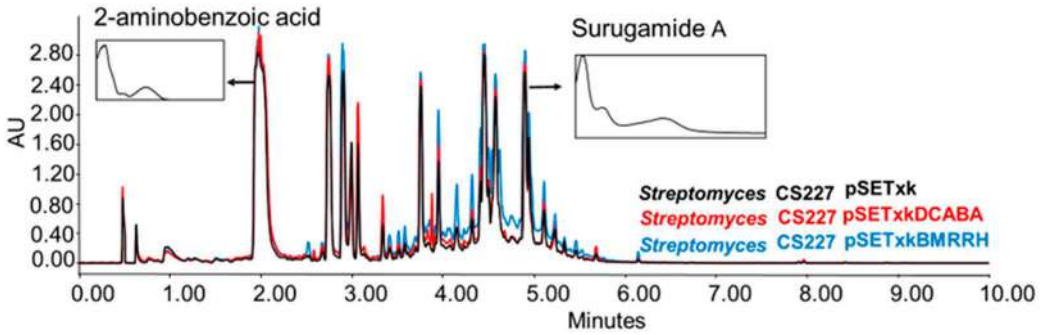
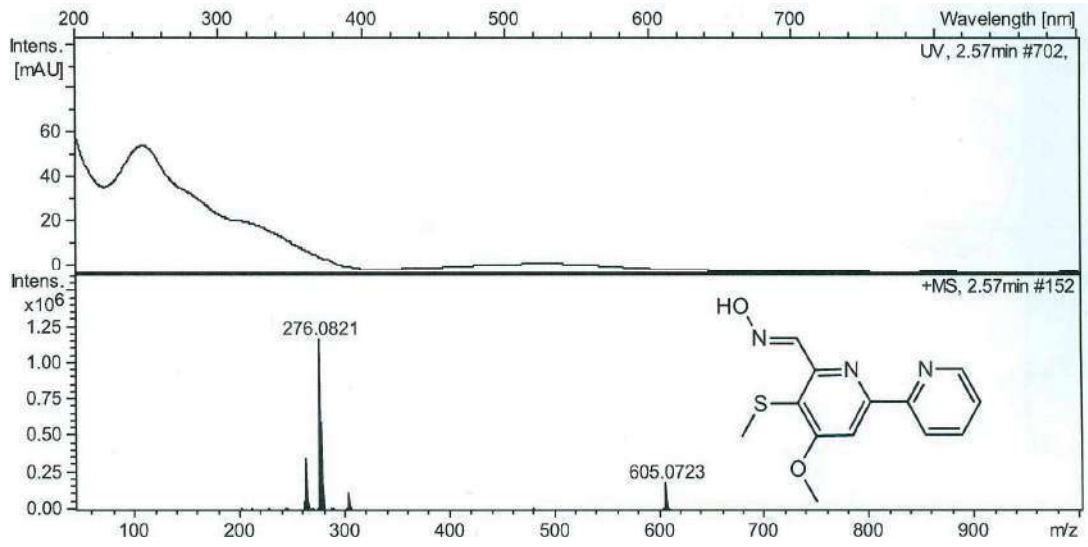


Figure S7. Comparative UPLC analysis of CS227 samples cultured in liquid SM17 medium and extracted with ethyl acetate: overproduction of 2-aminobezoic and surugamide by both strains can be observed. UV-Vis spectrums of these compounds are shown.

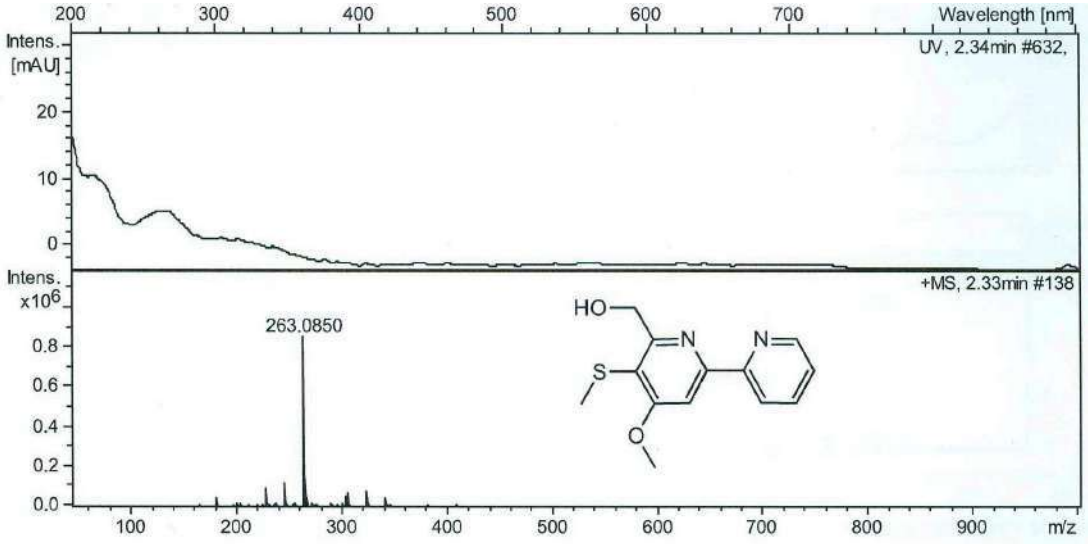
LC-MS dereplication: **Data corresponding to the identification of compounds whose production was activated in this work**

CS014:

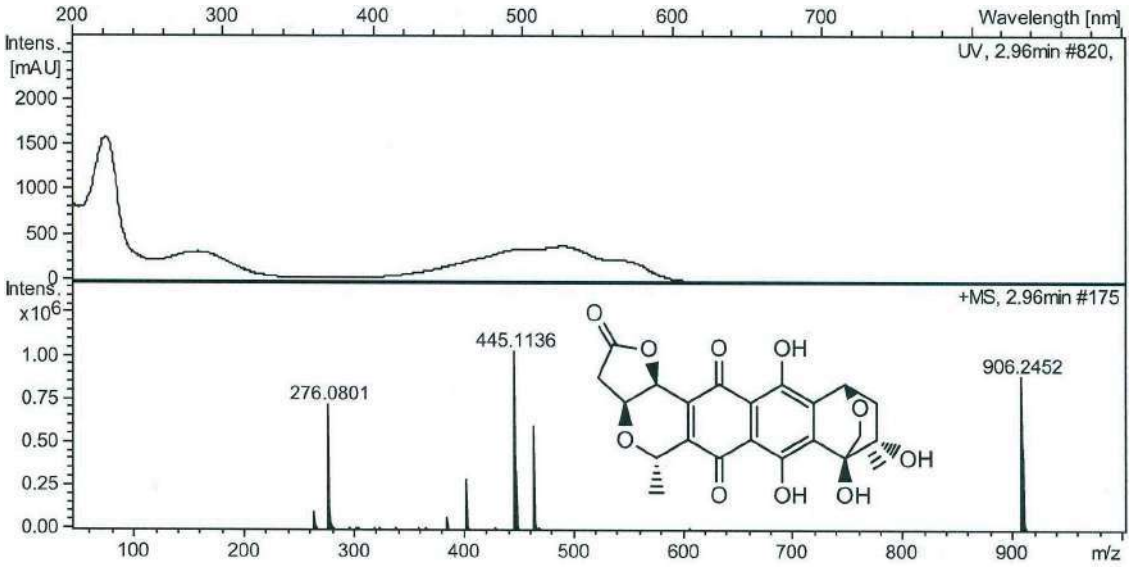
Collismycin A-B, $C_{13}H_{13}N_3O_2S$:



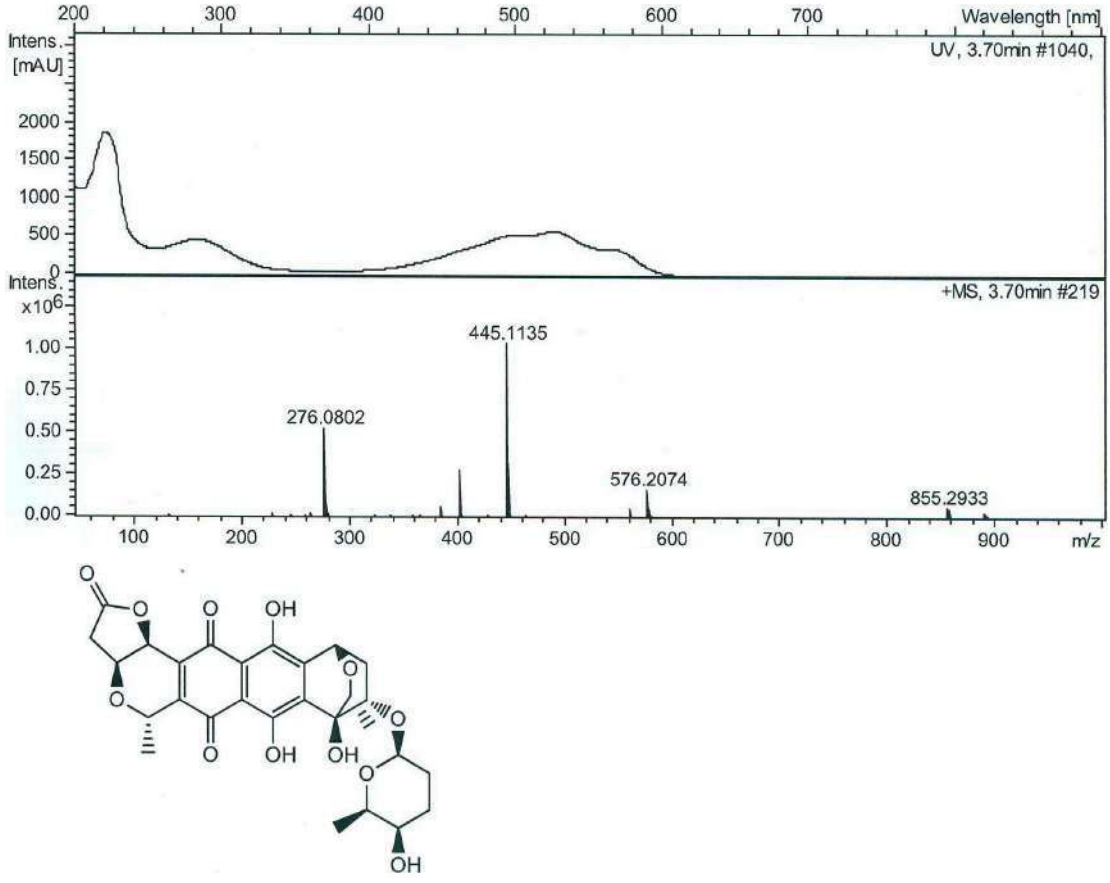
Collismycin C, $C_{13}H_{14}N_2O_2S$:



Granaticin A, $C_{22}H_{20}O_{10}$:

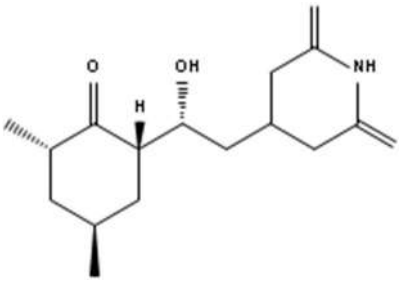


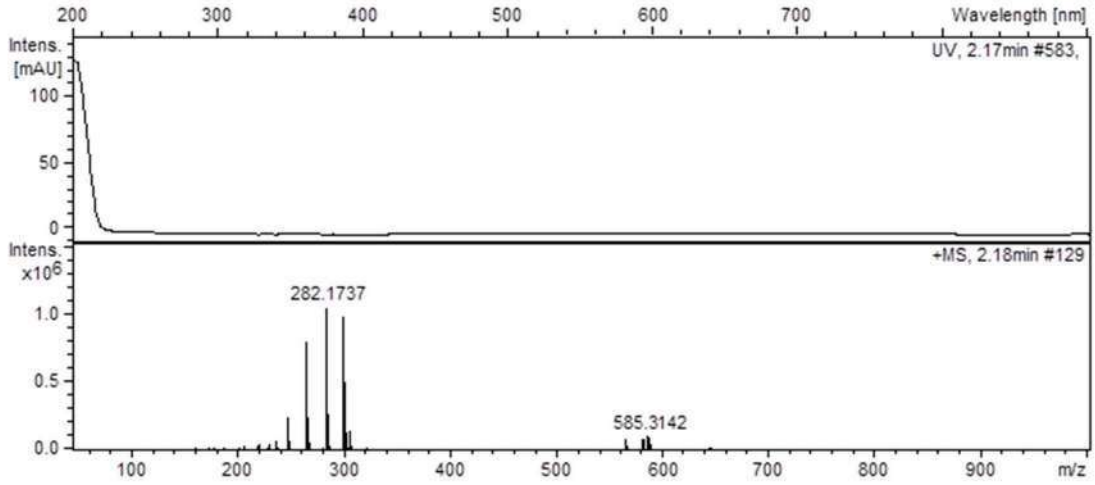
Granaticin C, $C_{28}H_{30}O_{12}$:



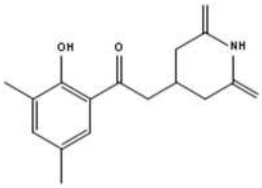
CS057:

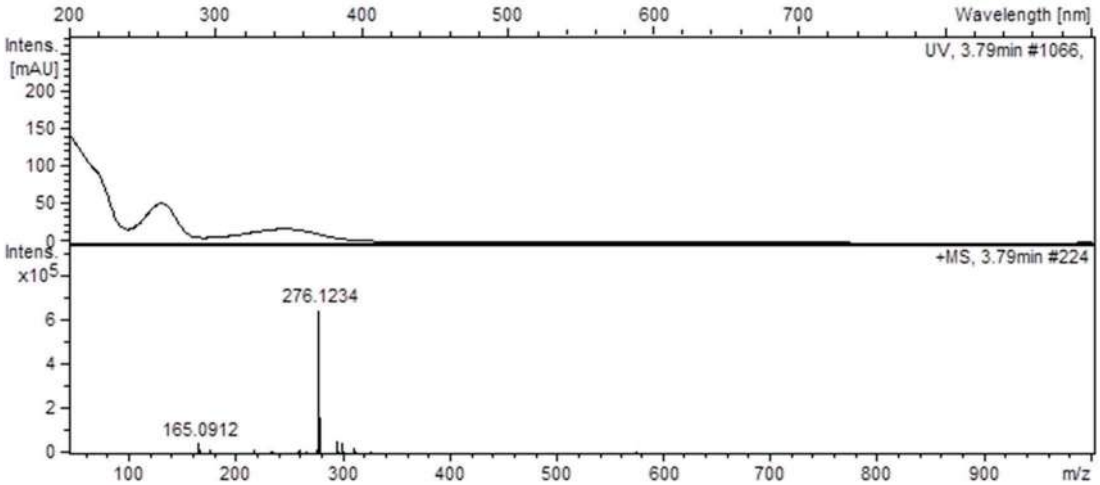
Cycloheximide, $C_{15}H_{23}NO_4$:



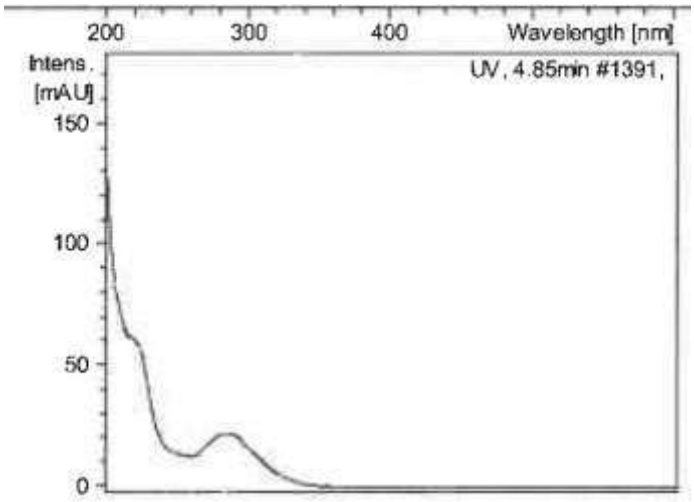


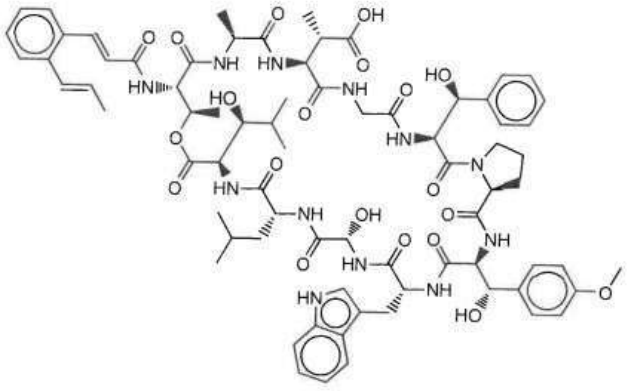
Actiphenol, $C_{15}H_{17}NO_4$:



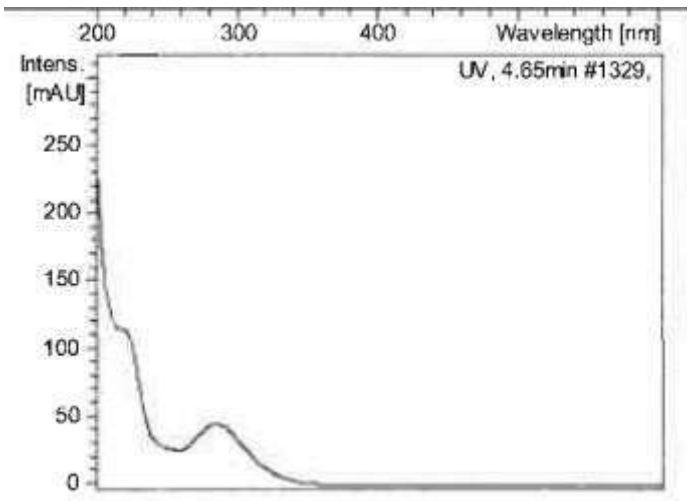


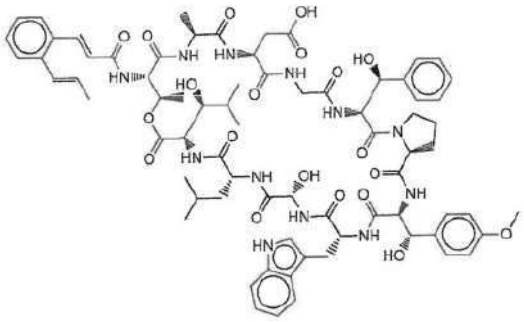
Skyllamycin A, $C_{75}H_{94}N_{12}O_{20}$





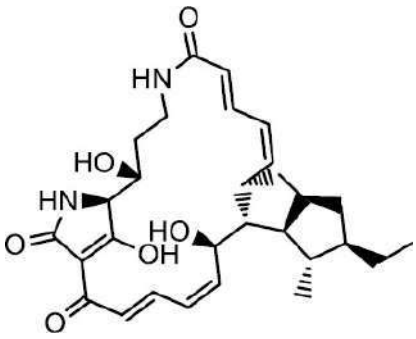
Skyllamycin B, $C_{74}H_{92}N_{12}O_{20}$

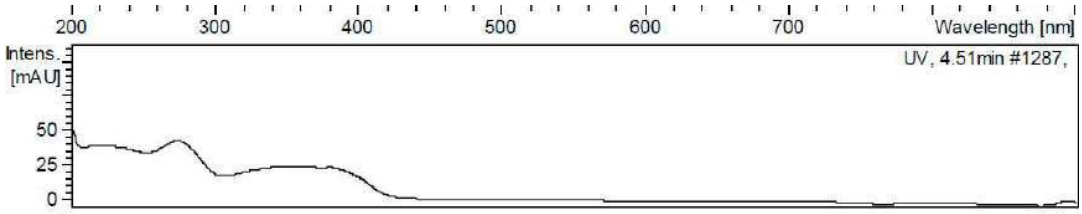


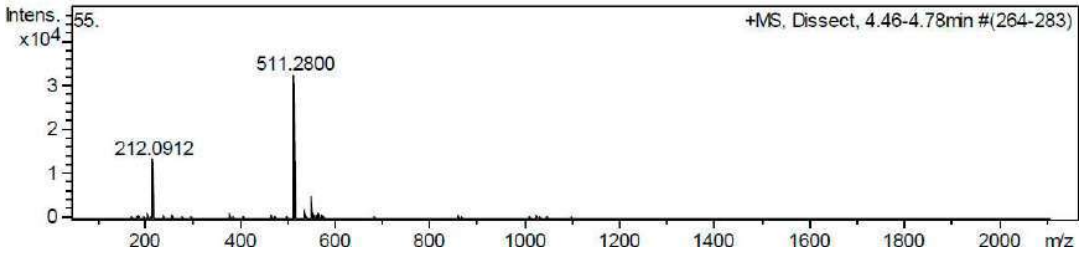


CS065a:

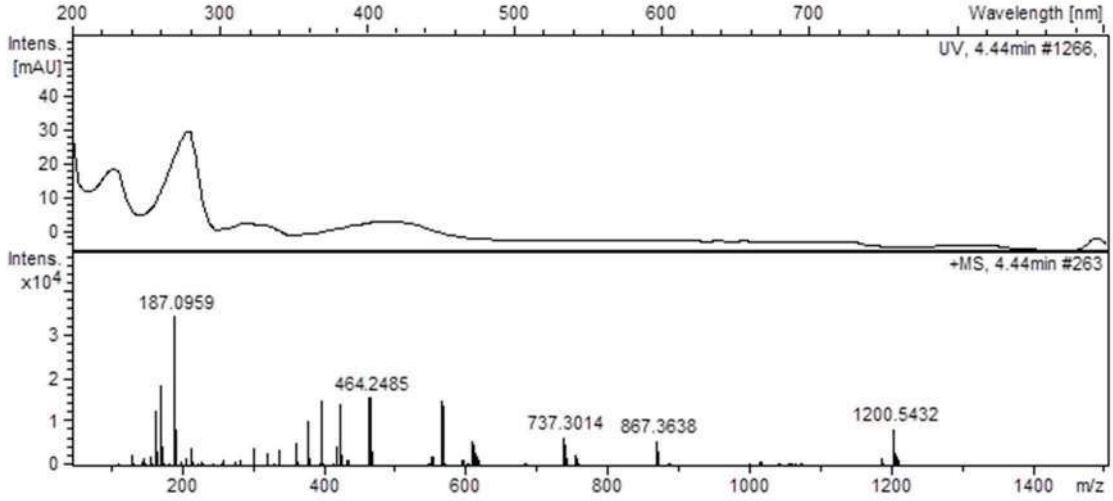
Alteramide A, $C_{29}H_{38}N_2O_6$:





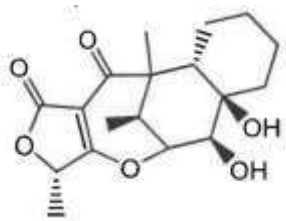


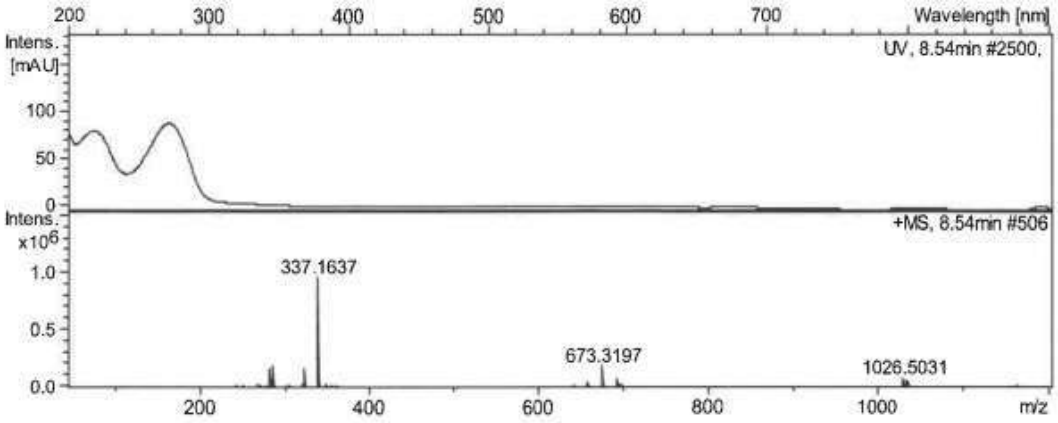
Chromomycin A3, $C_{57}H_{82}O_{26}$:



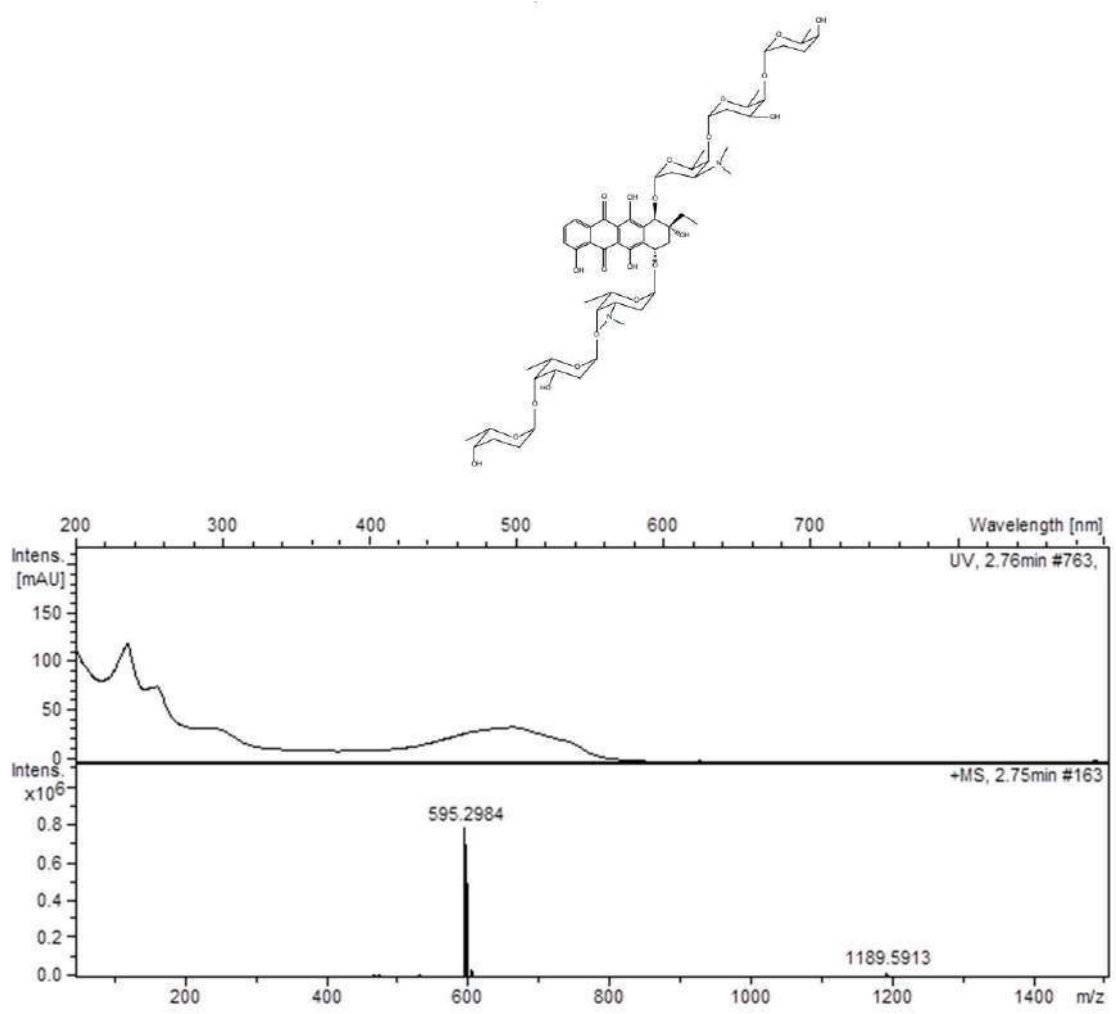
CS081a:

Dihydrotetrodecamycin, $C_{18}H_{24}O_6$:



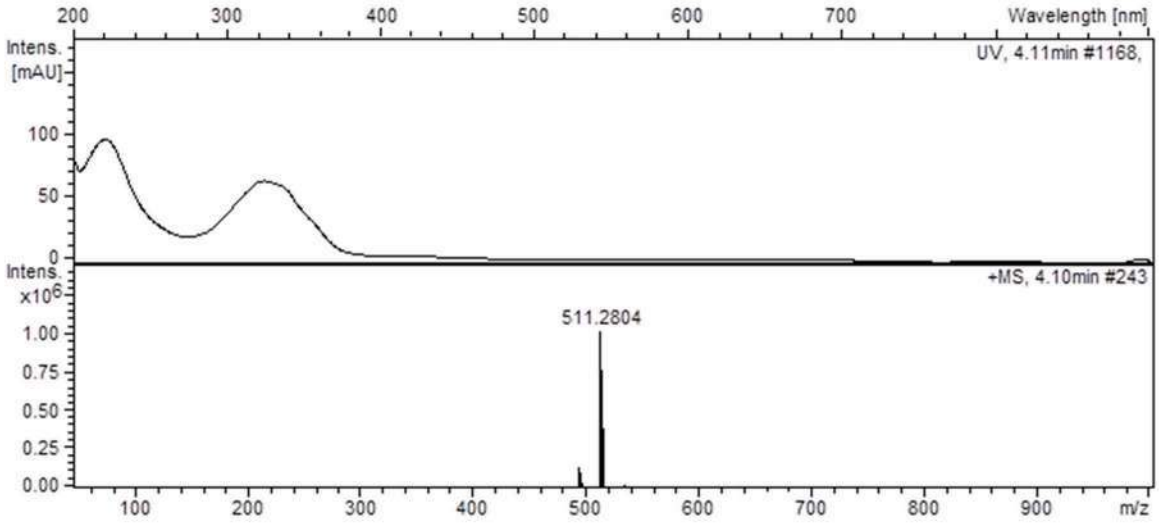


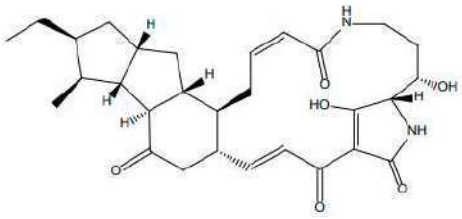
Cosmomycin D, $C_{60}H_{88}N_2O_{22}$:



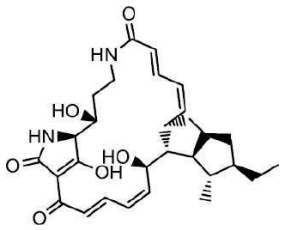
CS090a:

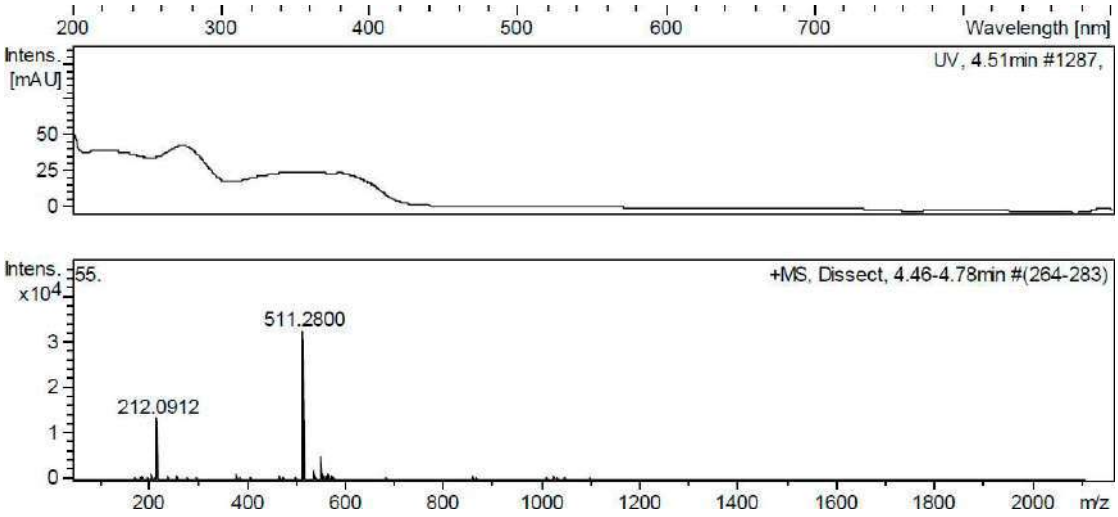
Maltophilin, $C_{29}H_{38}N_2O_6$:



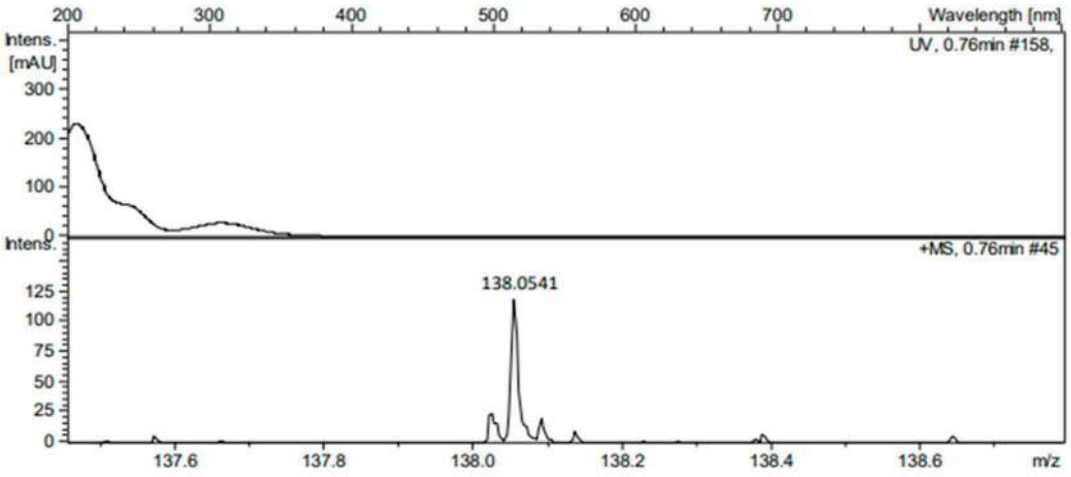


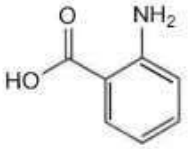
Alteramide A, $C_{29}H_{38}N_2O_6$:





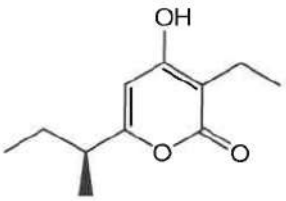
2-aminobenzoic acid, $C_7H_7NO_2$:

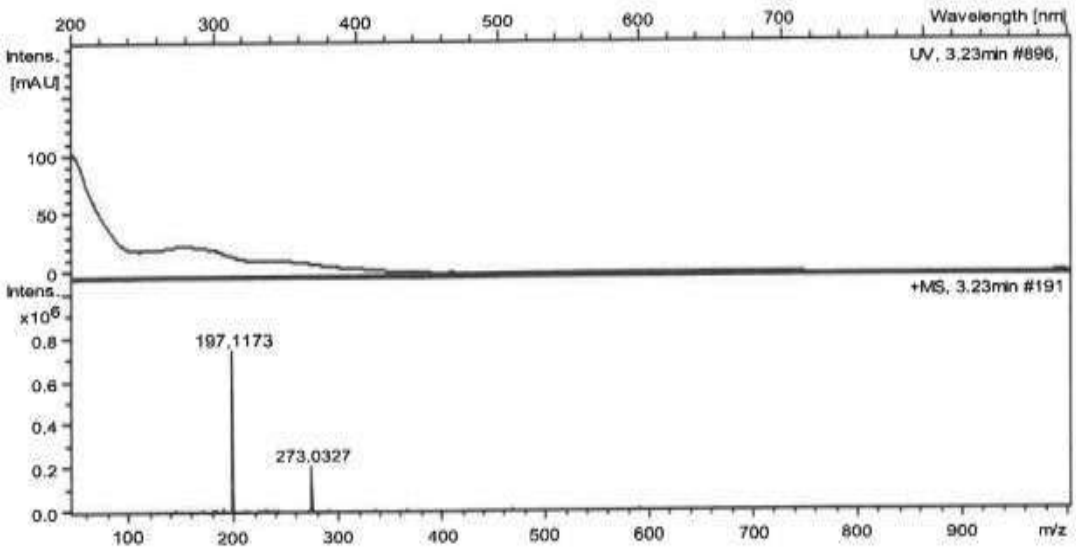




CS113:

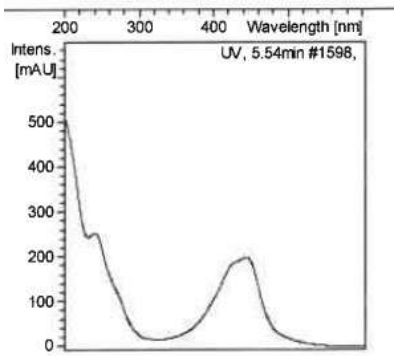
Germicidin A, $C_{11}H_{16}O_3$:

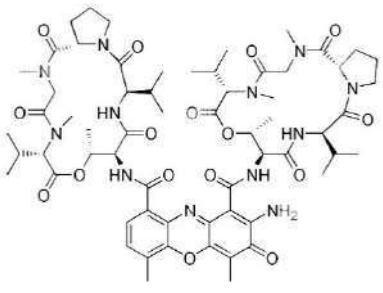




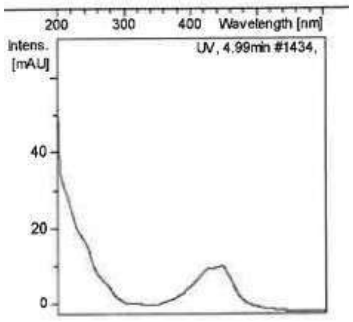
CS131:

Actinomycin D, $C_{62}H_{86}N_{12}O_{16}$:

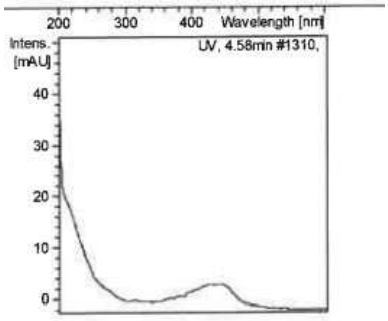




Actinomycin I, $C_{62}H_{86}N_{12}O_{17}$:

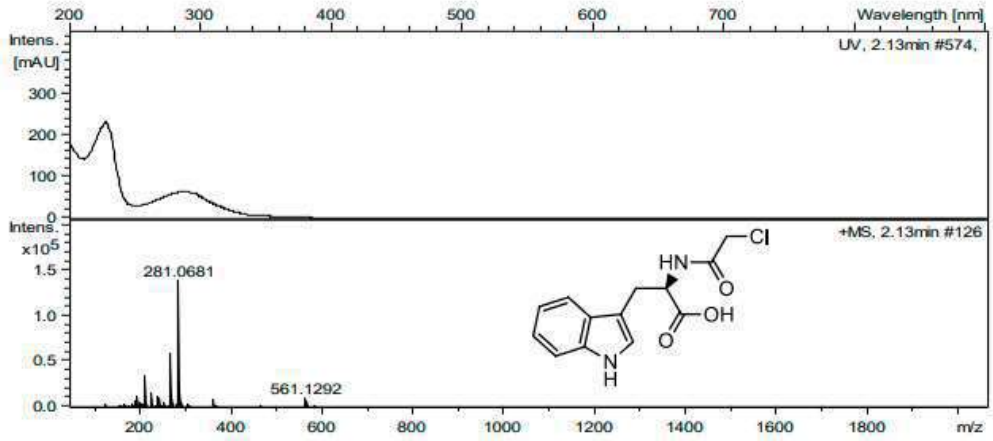


Actinomycin G4, $C_{61}H_{84}N_{12}O_{17}$:

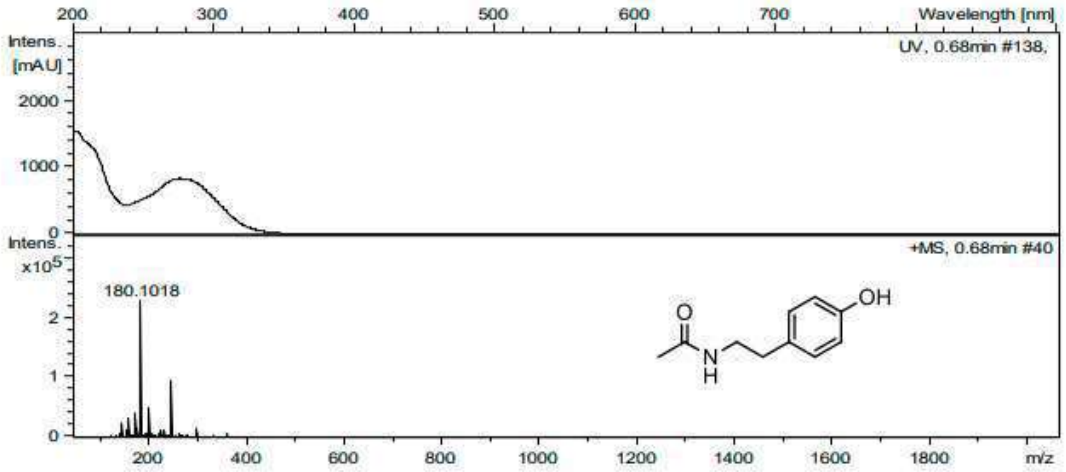


CS147:

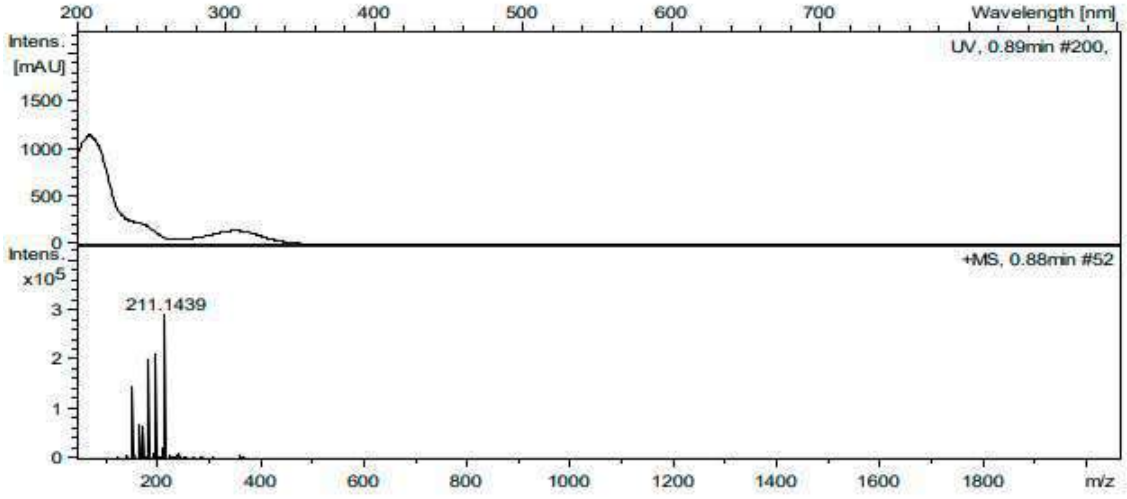
N-Chloroacetyl tryptophan, $C_{13}H_{13}ClN_2O_3$:



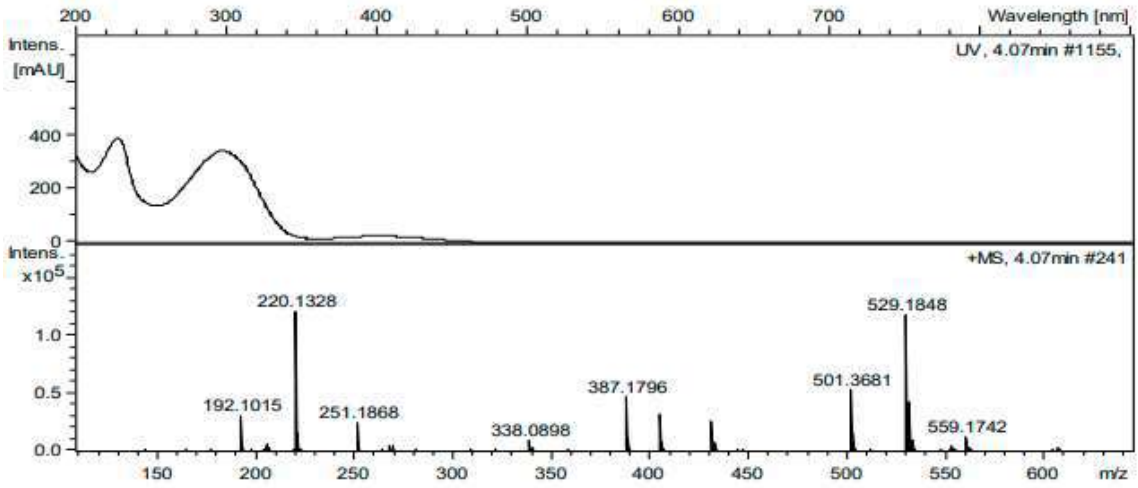
N-acetyltyramine, $C_{10}H_{13}NO_2$

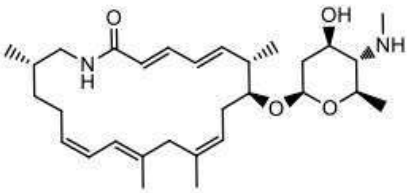


Cyclo (leu-pro), $C_{11}H_{18}N_2O_2$:

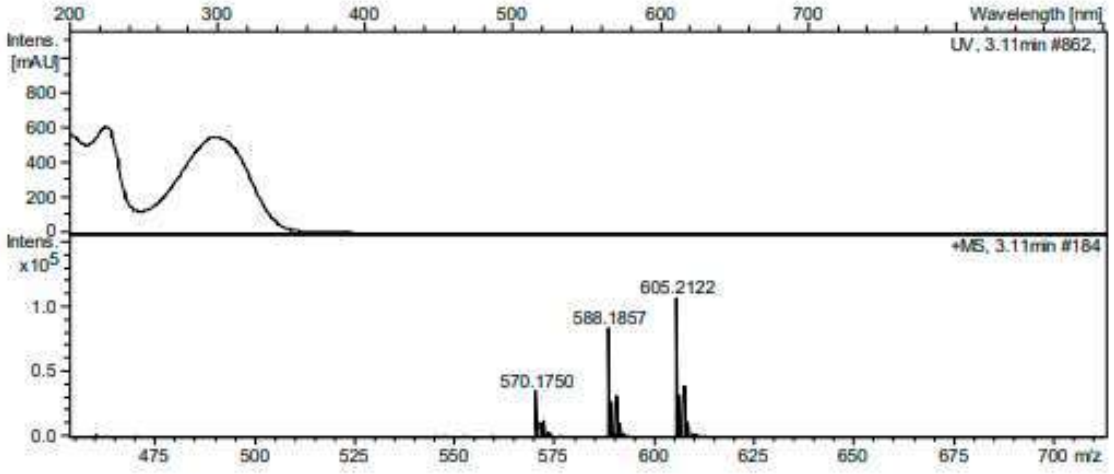


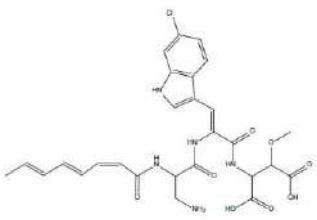
Vicenistatin, $C_{30}H_{48}N_2O_4$:



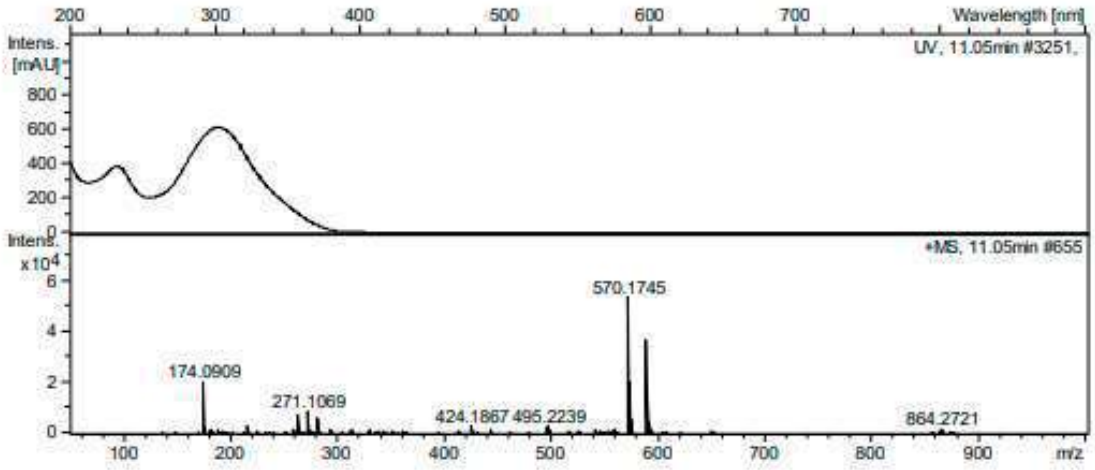


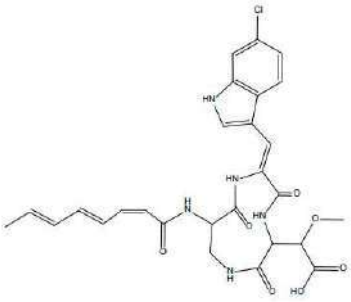
Colibrimycin A1, $C_{27}H_{30}N_5O_8$:



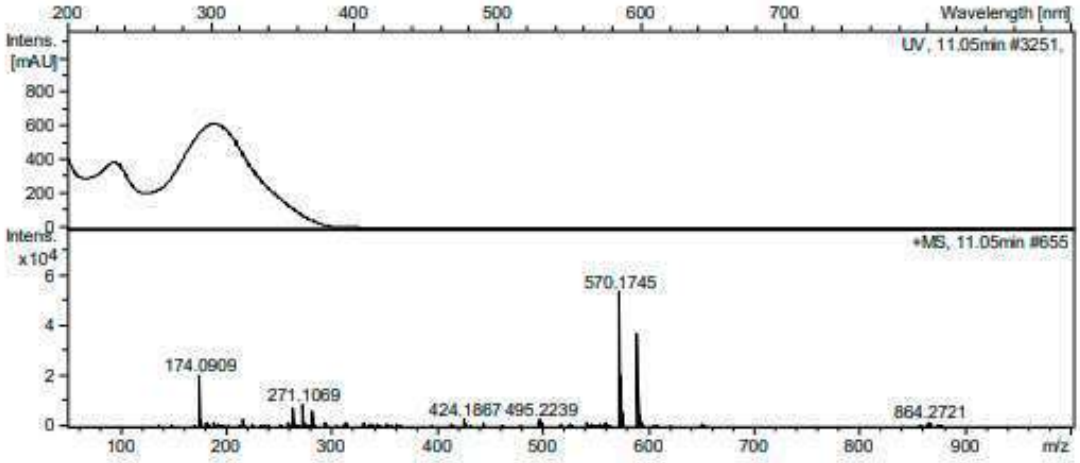


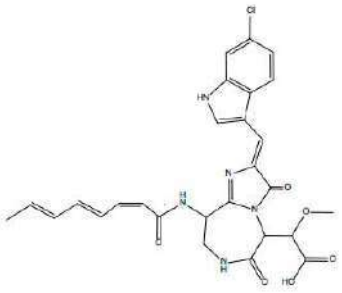
Colibrimycin A2, $C_{27}H_{28}ClN_5O_7$:



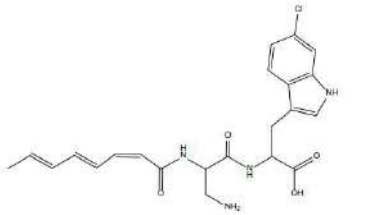


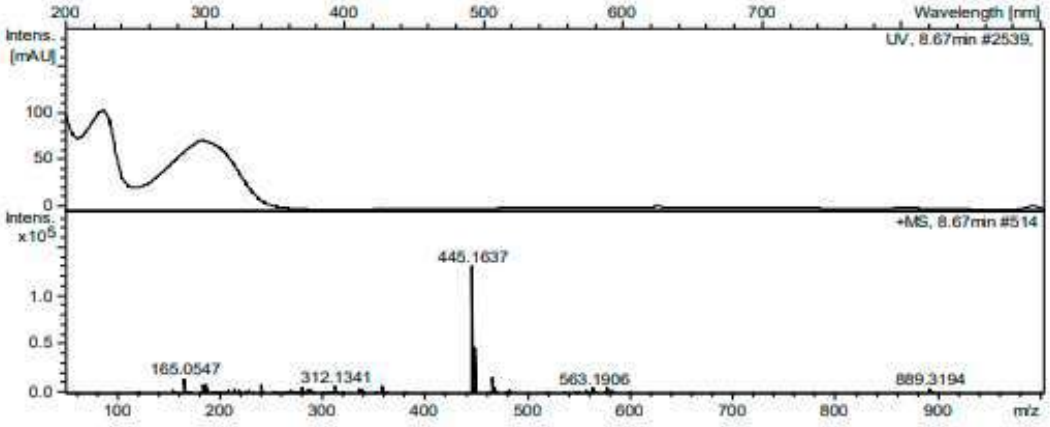
Colibrimycin A3, $C_{27}H_{28}ClN_5O_7$:



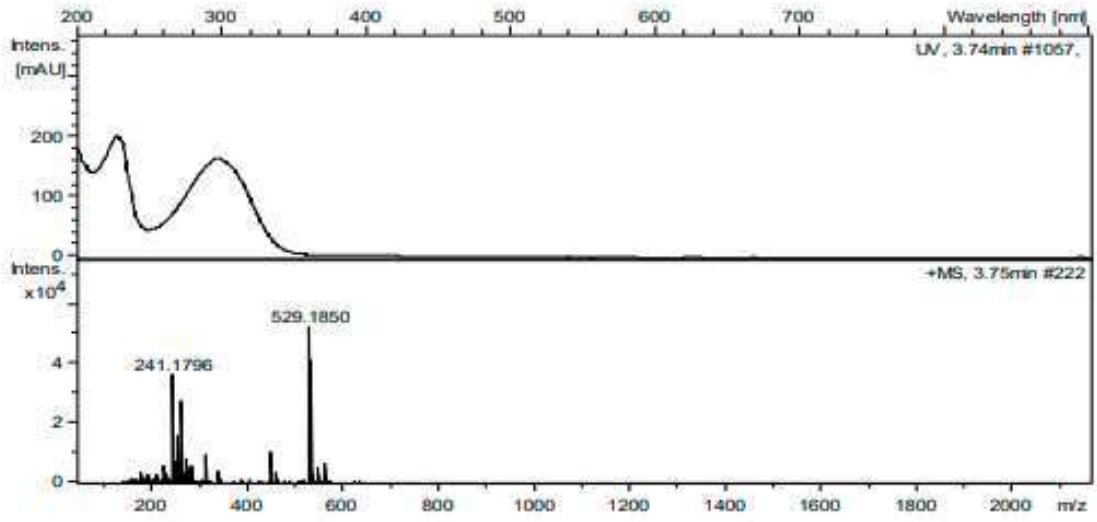


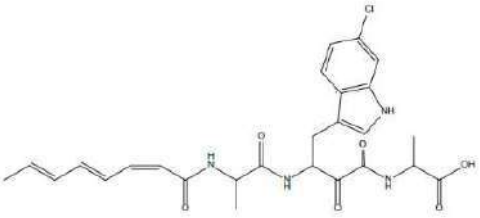
Colibrimycin A5, $C_{22}H_{25}ClN_4O_4$:



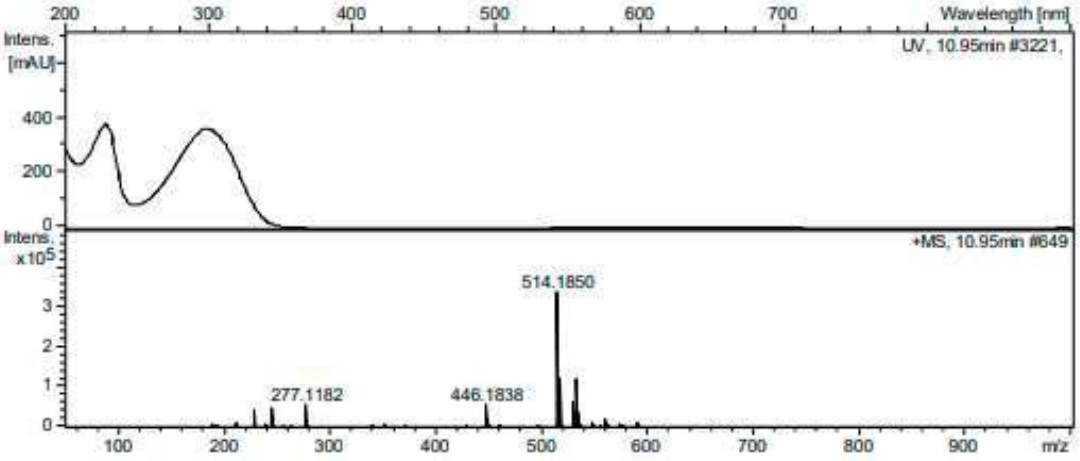


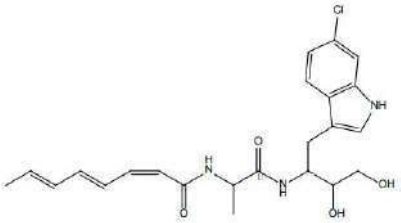
Colibrimycin C3, $C_{26}H_{29}ClN_4O_6$:



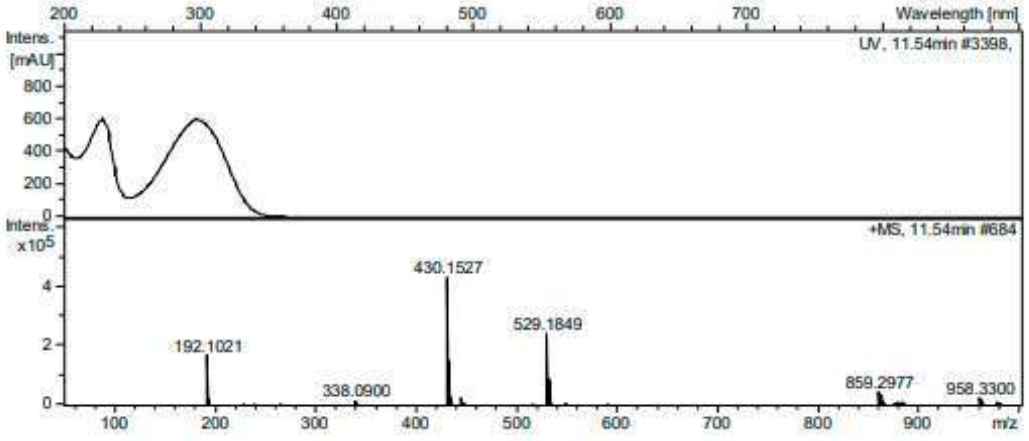


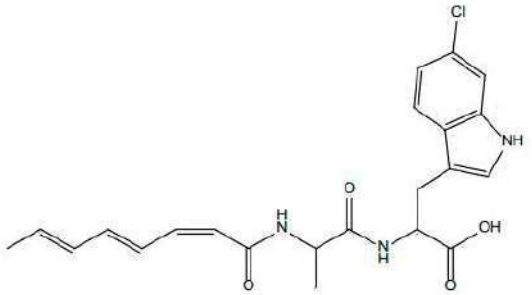
Colibrimycin C4, $C_{23}H_{28}ClN_3O_4$:





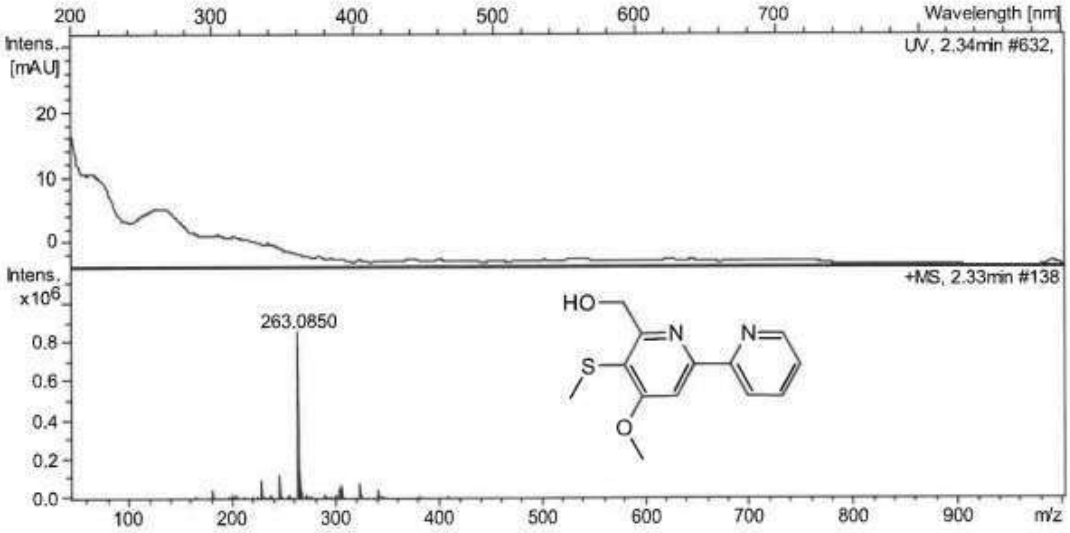
Colibrimycin C6, $C_{22}H_{24}ClN_3O_4$:





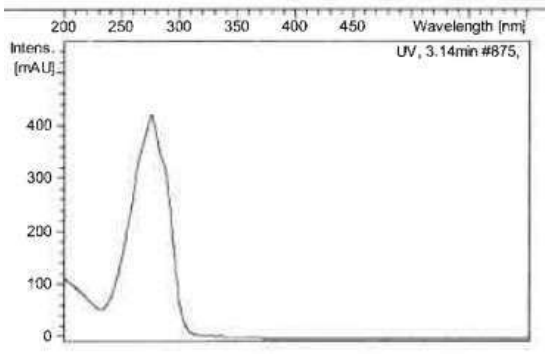
CS149:

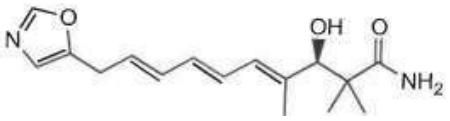
Collismycin C, $C_{13}H_{13}N_3O_2S$:



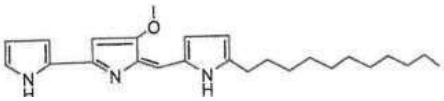
CS159:

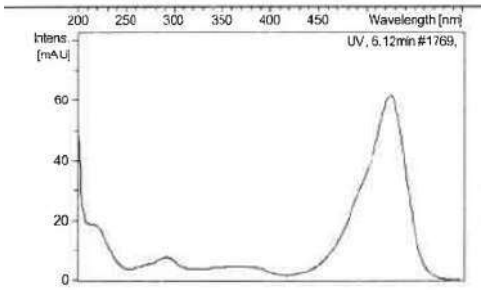
Inthomycin, $C_{16}H_{22}N_2O_3$:





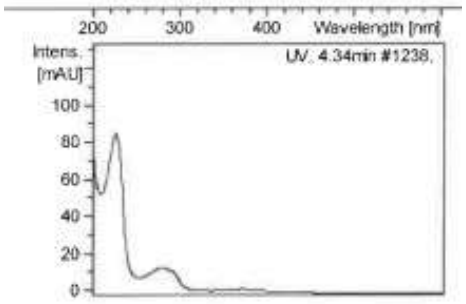
Undecylprodigiosin, $C_{25}H_{35}N_3O$:

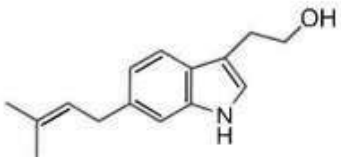




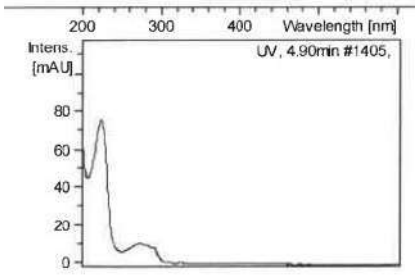
CS207:

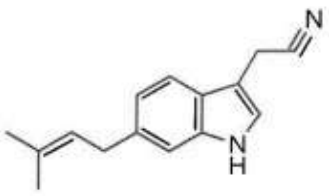
3-(2-Hydroxyethyl)-6-prenylindole, $C_{15}H_{19}NO$





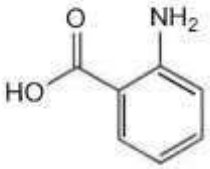
3-Cyanomethyl-6-prenylindole, $C_{15}H_{16}N_2$

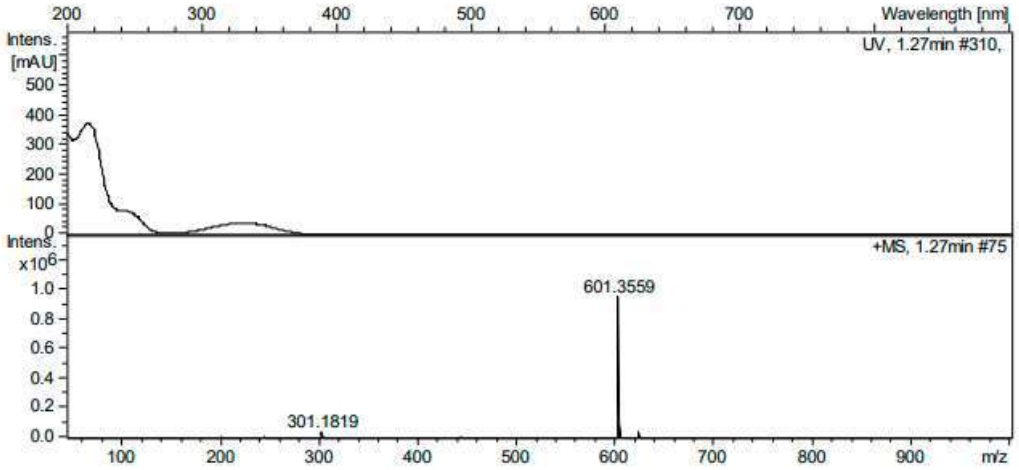




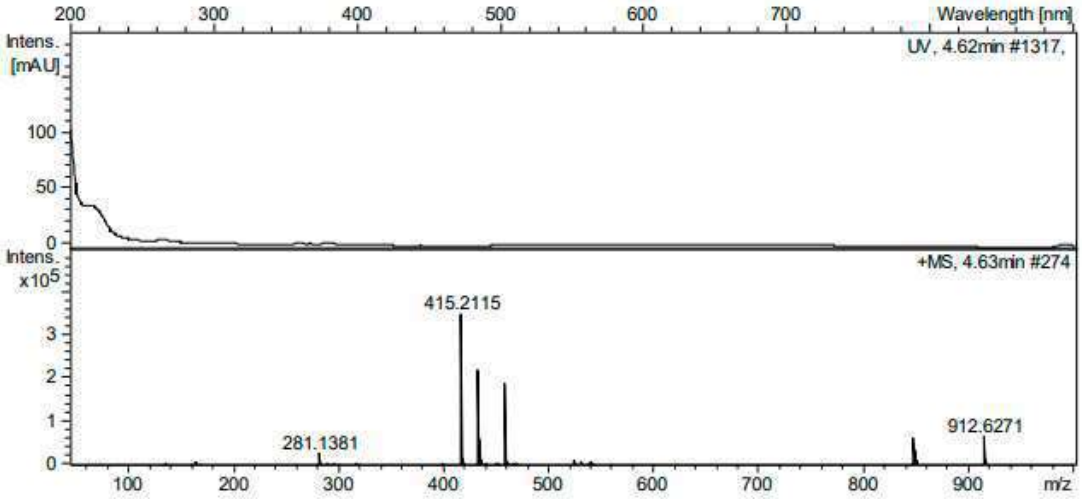
CS227:

2-Aminobenzoic acid, $C_7H_7NO_2$:



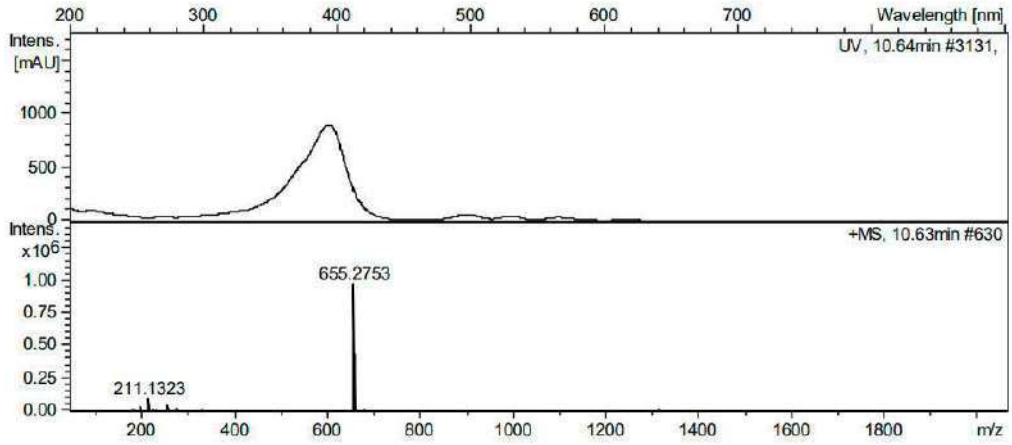


Suguramide A, $C_{48}H_{81}N_9O_8$:



Several strains:

Coproporphyrin, $C_{36}H_{38}N_4O_8$



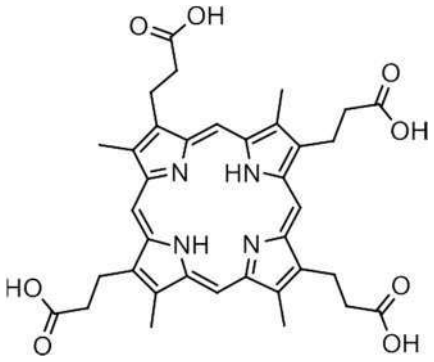


Table S4: Transcription Factor Binding Sites (TFBS) involved in this work find with strong confidence in the chromosome of each strain using AntiSMASH 7.0.

Strain	Cluster	Cluster type and MiBiG identification (over 65% similarity)	TFBS
CS014	1.22	Terpene (Geosmin 100%)	AbrC3
	1.23	Butyrolactone	AbrC3
	4.1	NRPS/PKS-I	AbrC3
CS057	1.7	PKS-I	AbrC3
	1.13	PKS-II/oligosaccharide/NRPS (warkmycin CS1/warkmycin CS2 97%)	AbrC3
CS065a	1.10	NRPS/NRPS-like	AbrC3
	1.15	nucleoside	AbrC3
	1.25	terpene	AbrC3
CS081a	1.3	Butyrolactone/NRPS	AbrC3
	4.7	PKS-II/terpene (Spore pigment 83%)	AbrC3
CS090a	1.7	Lanthipeptide –II/ Lanthipeptide -III	AbrC3
	1.15	Lanthipeptide –III (AmfS 100%)	AbrC3
	1.26	PKS-III/NRPS	AfsR
	1.28	NRPS/PKS-I/ Thiopeptide/ Linear azol(in)e-containing peptides/ RRE-element containing cluster	AbrC3
	2.1	Linear azol(in)e-containing peptides	AbrC3
	2.3	Terpene (Geosmin 100%)	AbrC3
CS113	1.4	RiPP-like	AbrC3
	1.19	NRPS-independent, IucA/IucC-like siderophores (Desferrioxamine 83%)	AbrC3
	1.22	PKS-II/PKS-like/oligosaccharide (Cervimycin 90%)	AbrC3
	1.25	Terpene	AfsR
	1.26	Butyrolactone/PKS-III (Germicidin 100%)	AfsR
			BldD
			AbrC3
CS131	1.2	Butyrolactone	AbrC3
	1.5	PKS-III (Naringenin 100%)	AbrC3
	1.6	NRPS-like/other (Actinomycin 89%)	AbrC3
	1.16	Lassopeptide (Stlassin 79%)	AbrC3
	1.25	Lanthipeptide -I	AfsR
	1.32	Terpene/PKS-I/NRPS (Isorenieratene 100%)	AbrC3
	1.37	NRPS/PKS-I	AbrC3
CS147	1.2	Butyrolactone	AbrC3
	1.5	PKS-III (Naringenin 100%)	AbrC3
	1.13	Phosphonate	AbrC3

Strain	Cluster	Cluster type and MiBiG identification (over 65% similarity)	TFBS
	1.31	NRPS/PKS-I	AbrC3
	1.32	Lassopeptide	AbrC3
			AfsR
CS149	1.12	Betalactone/Furan/Butyrolactone	AfsR
	1.13	Oligosaccharide/ Linear azol(in)e-containing peptides/ RRE-element containing cluster	AfsR
	1.31	NRPS/PKS-I	AbrC3
CS159	1.3	PKS-I/ PKS-like (arsono-polyketide 80%)	AbrC3
	1.10	NRP-metallophore/NRPS/PKS-I/NRPS-like/prodigiosin (Undecylprodigiosin 100%)	AbrC3
	1.18	PKS-II	AbrC3
	1.19	NI-siderophore (Desferroxiamin 83%)	AbrC3
	1.25	Indole (5-dimethylallylindole-3-acetonitrile 100%)	AfsR
CS207	1.2	PKS-II (Spore pigment 66%)	AbrC3
	1.9	Indole (5-dimethylallylindole-3-acetonitrile 100%)	AfsR
	1.12	PKS-II/Butyrolactone (Fluostatins M-Q 65%)	AfsR
	2.8	Lanthipeptide -III	AfsR
CS227	1.1	NRPS/NRPS-like	AfsR
	1.14	NRPS	AbrC3
	1.16	NRPS-independent, IucA/IucC-like siderophores (Desferroxiamin B 100%)	AbrC3

Table S5: TTA codons find in the chromosome of each strain using AntiSMASH 7.0.

Strain	Cluster	Type of cluster and MiBiG identification (over 65% similarity)
CS014	1.1	NRPS-like/NRPS
	1.3	PKS-I/NRPS (SGR PTMs 100%)
	1.4	Terpene (hopene 69%)
	1.5	NRPS (Holomycin 92%)
	1.6	NRPS/ PKS-I (Collismycin A 77%)
	1.8	Oligosacaride/PKS-I (Sipanmycin 100%)
	1.10	Terpene
	1.11	Lanthipeptide-III (AmfS 100%)
	1.16	Lanthipeptide-III/ Lanthipeptide-II
	1.20	PKS-III (Naringenin 100%)
	1.22	Terpene (Geosmin 100%)
	1.24	NRPS-like
	2.1	NRPS/ PKS-I
	2.4	Thiopeptide/ Linear azol(in)e-containing peptides
	2.5	NRPS/PKS-III
	4.1	NRPS/PKS-I
CS057	1.1	NRPS/PKS-I
	1.2	NRPS-like/lanthipeptide- IV/ transAT-PKS (Cycloheximide 94%)
	1.3	NRPS/PKS-III

Strain	Cluster	Type of cluster and MiBiG identification (over 65% similarity)
	1.6	NRPS/PKS-I
	1.7	PKS-I
	1.8	RiPP-like
	1.9	NRPS/NRPS-like (SGR PTMs 100%)
	1.11	Terpene (Hopene 69%)
	1.13	PKS-II/oligosaccharide/NRPS (warkmycin CS1/warkmycin CS2 97%)
	1.15	Terpene
	1.16	Lanthipeptide-III (AmfS 100%)
	1.20	Ectoine/butyrolactone/ladderane/arylpolyene/NRPS/PKS-I (Skyllamycins 97%)
	1.23	Lanthipeptide-III/ Lanthipeptide-II
	1.28	Thiopeptide/ Linear azol(in)e-containing peptides
	1.29	PKS-III (Narigenin 100%)
	1.31	Terpene (Geosmin 100%)
	1.32	Butyrolactone
CS065a	1.6	NRPS/PKS-I (Malthophilin 100%)
	1.7	NRPS
	1.8	Terpene (hopene 69%)
	1.9	arylpolyene/NRPS-like/ectoine
	1.10	NRPS-like/ NRPS
	1.12	Terpene
	1.13	Lanthipeptide-III (Amfs 100%)
	1.15	Nucleoside
	1.17	NRPS/PKS-I
	1.19	NRP-metallophore/NRPS/PKS-like
	1.20	Thiopeptide/ Linear azol(in)e-containing peptides
	1.22	Lanthipeptide-III/ Lanthipeptide-II
	1.23	PKS-II/oligosaccharide (Chromomycin 100%)
	1.26	PKS-III
	2.2	Terpene (Geosmin 100%)
CS081a	1.3	Butyrolactone/NRPS
	1.4	PKS-II/oligosaccharide/PKS-like (Cosmomycin D 97%)
	1.5	Butyrolactone/furan
	1.9	NRPS
	2.1	Lassopeptide (Albusnodin 100%)
	4.1	Lanthipeptide-III
	4.3	NRPS
	4.5	Terpene (Geosmin 100%)
	4.6	NRP-metallophore/NRPS
	4.9	PKS-I
	5.1	Phenazide
CS090a	1.1	PKS-III (Naringenin 100%)
	1.2	NRPS-like/NRPS-betalactone
	1.3	NRPS-like/terpene (2-methylisoborneol 100%)
	1.6	PKS-II/oligosaccharide
	1.7	Lanthipeptide-II/ Lanthipeptide-III
	1.9	NRPS-like
	1.10	Thiopeptide/ Linear azol(in)e-containing peptides
	1.12	PKS-I

Strain	Cluster	Type of cluster and MiBiG identification (over 65% similarity)
	1.15	Lanthipeptide-III (AmfS 100%)
	1.16	Terpene
	1.18	Butyrolactone
	1.19	Lanthipeptide-I
	1.20	Terpene (Hopene 69%)
	1.21	NRPS/PKS-I (Maltophilin 100%)
	1.23	NRPS
	1.24	NRPS/RiPP-like
	1.26	PKS-III/NRPS
	1.28	NRPS/PKS-I/thiopeptide/ Linear azol(in)e-containing peptides/ RRE-element containing cluster
	2.3	Terpene (Geosmin 100%)
	2.4	PKS-like/PKS-I/NRPS/NRP-metallophore/ transAT-PKS (Griseobactin 100%)
	4.1	PKS-III
	5.2	PKS-like
CS113	1.1	PKS-I/NRPS/NRP –metallophore/NRPS-like/other
	1.2	Other
	1.12	PKS-I/ NRPS-like/prodigiosin (Undecylprodigiosin 100%)
	1.14	NRPS
	1.16	Terpene (Albaflavenone 100%)
	1.26	Butyrolactone/ PKS-III (Germicidin 100%)
	1.27	Terpene (Isorenieratene (71%)
	1.28	Indole (5-dimethylallylindole-3-acetonitrile 100%)
CS131	1.1	NRPS-like
	1.3	Terpene (Geosmin 100%)
	1.6	NRPS-like/other (Actinomycin 89%)
	1.7	Terpene (Isorenieratene 87%)
	1.8	NRPS/ NRPS-like
	1.9	Terpene
	1.11	Lanthipeptide-II/ Lanthipeptide-III
	1.12	NRPS-independent, IucA/IucC-like siderophores (Desferroxiamin B 100%)
	1.14	NRPS-like
	1.15	NRPS
	1.16	Lassopeptide (Stlassin 79%)
	1.17	Lanthipeptide-II
	1.18	NRPS/Ladderane/arylpolyene/NRPS-like/Lassopeptide
	1.21	Lanthipeptide-III (AmfS 100%)
	1.23	Terpene
	1.25	Lanthipeptide-I
	1.26	NRPS (Holomycin 92%)
	1.29	Terpene (Hopene 69%)
	1.30	PKS-I/NRPS (SGR PTMs 100%)
	1.32	Terpene/PKS-I/NRPS (Isorenieratene 100%)
	1.35	thiopeptide/ Linear azol(in)e-containing peptides
	1.36	PKS-III/NRPS
	1.38	PKS-I (Stambomycins 96%)
CS147	1.1	NRPS-like

Strain	Cluster	Type of cluster and MiBiG identification (over 65% similarity)
	1.3	Terpene (Geosmin 100%)
	1.4	NRP-metallopore, NRPS, NRPS-like (Griseobactin 100%)
	1.5	PKS-III (Naringenin 100%)
	1.6	Arylpolyene
	1.7	Terpene (Isorenieratene 87%)
	1.10	Lanthipeptide-II/ Lanthipeptide-III
	1.13	Phosphonate
	1.16	Ladderane/NRPS (Colibrimycin (75%)
	1.17	PKS-I/ RRE-element containing cluster (Vicenistatin 100%)
	1.19	Lanthipeptide-III (AmfS 100%)
	1.20	Terpene
	1.22	NRPS (Holomycin 92%)
	1.24	Terpene (Hopene 69%)
	1.25	NRPS/PKS-I/RiPP-like (SGR PTMs 100%)
	1.26	Terpene/ NRPS/PKS-I (Isorenieratene 100%)
	1.27	RiPP-like (Streptamidine 66%)
	1.32	Lassopeptide
CS149	1.1	NRPS-like
	1.3	Terpene (Geosmin 100%)
	1.5	Terpene (Isorenieratene 100%)
	1.6	PKS-III (Narigenin 100%)
	1.10	Lanthipeptide-II/ Lanthipeptide-III
	1.12	Betalactone/Furan/Butyrolactone
	1.16	Lanthipeptide-III (AmfS 100%)
	1.17	NRPS/ NRPS-like
	1.18	Terpene
	1.20	Oligosaccharide/PKS-I (Sipanmycin 100%)
	1.21	NRPS/PKS-I (Collismycin A 70%)
	1.22	NRPS (Holomycin 92%)
	1.25	NRPS/PKS-I/RiPP-like (SGR PTMs 100%)
	1.26	NRPS/PKS-I
	1.29	thiopeptide/ Linear azol(in)e-containing peptides
CS159	1.5	Trans-AT PKS/NRPS/NRPS-like (Inthomycin B 100%)
	1.8	Betalactone
	1.10	NRP-metallophore/NRPS/PKS-I/NRPS-like/prodigiosin (Undecylprodigiosin 100%)
	1.11	N-siderophore
	1.12	PKS-II (Spore pigment 66%)
	1.13	Terpene (Albaflavenone 100%)
	1.16	Other
	1.17	NRPS (Sarpeptin 91%)
	1.18	PKS-II
	1.20	Melanin
	1.22	PKS-III (Flaviolin/1,3,6,8-tetrahydroxynaphthalene 100%)
	1.23	PKS-III (Germicidin 100%)
	1.25	Indole (5-dimethylallylindole-3-acetonitrile 100%)
	1.26	Terpene (2-methylisoborneol 100%)
	2.1	PKS-II/Butyrolactone
CS207	1.2	PKS-II (Spore pigment 66%)

Strain	Cluster	Type of cluster and MiBiG identification (over 65% similarity)
	1.6	PKS-III (Flaviolin/1,3,6,8-tetrahydroxynaphthalene 100%)
	1.7	NRPS-like (Streptothricin 95%)
	1.8	terpene
	1.9	Índole (5-dimethylallylindole-3-acetonitrile 100%)
	1.10	terpene
	1.11	Indole (7-prenylisatin 83%)
	1.12	PKS-II/Butyrolactone (Fluostatins M-Q 65%)
	2.22	NRPS (CDA 72%)
CS227	1.1	NRPS-like/ NRPS
	1.4	NRPS/ PKS-I (SGR PTMs 100%)
	1.7	PKS-I/ Terpene
	1.9	Terpene (Geosmin 100%)
	1.14	NRPS
	1.20	PKS-I/NRPS/Lanthipeptide-II/transAT-PKS/NRPS-like (Antimycin 100%)

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Capítulo II

Volatile Compounds in Actinomycete Communities: A New Tool for Biosynthetic Gene Cluster Activation, Cooperative Growth Promotion, and Drug Discovery

cells

MDPI

Article

Volatile Compounds in Actinomycete Communities: A New Tool for Biosynthetic Gene Cluster Activation, Cooperative Growth Promotion, and Drug Discovery

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Abstract: The increasing appearance of multiresistant pathogens, as well as emerging diseases, has highlighted the need for new strategies to discover natural compounds that can be used as therapeutic alternatives, especially in the genus *Streptomyces*, which is one of the largest producers of bioactive metabolites. In recent years, the study of volatile compounds (VOCs) has raised interest because of the variety of their biological properties in addition to their involvement in cell communication. In this work, we analyze the implications of VOCs as mediating molecules capable of inducing the activation of biosynthetic pathways of bioactive compounds in surrounding Actinomycetes. For this purpose, several strains of *Streptomyces* were co-cultured in chamber devices that allowed VOC exchange while avoiding physical contact. In several of those strains, secondary metabolism was activated by VOCs emitted by companion strains, resulting in increased antibiotic production and synthesis of new VOCs. This study shows a novel strategy to exploit the metabolic potential of Actinomycetes as well as emphasizes the importance of studying the interactions between different microorganisms sharing the same ecological niche.

Keywords: *Streptomyces*; volatile compounds; biosynthetic potential; secondary metabolites; rumycins

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1. Introduction

The pandemic caused by SARS-Cov2 has only highlighted something that has been known for several decades: the lack of new bioactive compounds useful not only to treat diseases caused by emerging pathogens but also to treat those due to pathogens resistant to conventional treatments. The World Health Organization (WHO) declared antimicrobial resistance one of the top 10 global public health threats facing humanity in 2019 and urged the scientific community to focus on new drug research and development [1].

Most anti-infective drugs are of natural origin, mainly produced by bacteria (with special mention to Actinomycetes) and fungi. In natural product research, re-isolation of already known compounds is a major bottleneck, therefore new approaches need to be followed [2,3]. The search for antibiotic producers in underexplored environments or mutualistic relationships with other organisms has demonstrated its usefulness. Marinopyrrole A and abyssomicin C are examples of natural drugs produced by marine bacteria with potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA) [4,5] and polyketides cyphomycin and sipanmycin isolated from the microbiome of leaf-cutter ants of the *Attini* tribe have demonstrated antifungal and antibacterial properties, respectively [6,7].

The development of new genomic techniques has revitalized the field of drug discovery as it has revealed the enormous biosynthetic potential of some microorganisms, such as Actinomycetes. In past years, the sequencing of the genome of a vast amount of Streptomycetes has pointed out the presence of a higher number of biosynthetic gene clusters (BGCs) for the production of secondary metabolites than initially thought. Many of these BGCs

are not expressed under laboratory conditions; thus, their products remain unknown. The induction of the expression of these silent BGCs is an attractive research field in drug discovery and efforts have been focused mainly on five approaches: (i) genetic manipulation of global or cluster-specific transcriptional regulators; (ii) expression of natural or synthetic BGCs in native or heterologous hosts; (iii) ribosomal engineering; (iv) OSMAC approaches through the systematic variation of growth parameters; and (v) mimicking the ecological environment of the producer strain via co-culture with other microorganisms or via the use of chemical elicitors (e.g., rare earth elements, dimethyl sulfoxide, ethanol) [8–13].

Traditionally, research on natural products was based on water-diffusible compounds, but in recent years, the importance of volatile natural compounds with interesting bioactivities has attracted the attention of the scientific community. Recently, Liu and co-workers applied a machine-learning workflow called MSHub/GNPS [14] to 37 different *Streptomyces* isolates and discovered the production of 581 different volatile compounds, which pointed out the high capacity of this bacterial genus to synthesize this kind of metabolite [15]. Volatile compounds (VOCs) are chemically diverse metabolites with common features: low molecular mass, high vapor pressure, low boiling point, and lipophilic nature [16]. In complex microbial environments, inter- and intra-species relationships are essential to maintain the balance of the community, and secondary metabolites play a key role in this equilibrium, as they participate in signaling, antibiosis, or competition between species [17–20]. As stated above, bacteria in mutualistic relationships with leaf cutter ants are an important source of new drugs. These ants collect leaves within their nests to cultivate the basidiomycete *Leucoagaricus gongylophorus*, which processes plant material, providing a rich source of nutrients for the ants [21]. This fungal garden is threatened by the pathogenic fungus *Escovopsis weberi,* and to fight against it and protect the stability of the nest, the ants have developed a complex microbiome composed of antimicrobial-producing Actinomycetes (mainly *Pseudonocardia* spp. and *Streptomyces* spp.) [22]. The production of specialized diffusible bioactive metabolites (e.g., candicidin D, antimycins, selvamycin) by these mutualistic bacteria has been reported [23]. In a confined space with many chambers filled with air (as is the case of those nests), it is logical to think that volatile compounds would play a significant role in this war. In 2021, Dhodary and Spiteller described the antifungal properties of inorganic ammonia produced by *Streptomyces* symbionts on *Escovopsis* sp. through the alkalization of the medium [24]. However, what about VOCs? Could they exert direct antibiotic activity or stimulate other microorganisms to produce antibiotics?

In this work, we tested the hypothesis that VOCs emitted by Actinomycetes isolated from leaf-cutter ants could serve as communication signals capable of activating the production of bioactive compounds by other Actinomycetes within the community. Accordingly, we confronted thirteen different Actinomycete strains isolated from the surface of the *Attini* ants (CS strains [7]) in solid culture using a specialized device designed to study the effect of the VOCs produced in the system (VOC chamber [25]). Using a bioassay-guided screening approach, we detected the activation or overproduction of chemically diverse bioactive natural compounds by Actinomycetes (e.g., cycloheximide, collismycin, cosmomycins, rumycins) when they were exposed to VOCs from other related species. Thus, the important role of volatiles as signaling agents and their usefulness for drug discovery studies were addressed.

2. Materials and Methods

2.1. Strains and Culture Conditions

The Actinomycete strains used in this work belong to an in-lab CS collection isolated from the cuticle of leafcutter ants from the tribe *Attini* [7,26]. Strains were routinely grown on MA plates [27] and incubated at 28 °C for 7 days. For metabolite production, strains were grown on agar plates of R5A [28], soy flour mannitol (SFM; [29]) or YMA (yeast extract 3 g; malt extract 3 g; peptone 5 g and glucose 10 g per liter).

2.2. Dual-Culture Actinomycetes in VOC Chambers

Actinomycete strains were grown on R5A or SFM plates at 28 °C occupying a surface of 16 cm^2 in the central part of the plates so that the edges of the colonies could be observed. After 24 h, the VOC chamber (J.D. Catalán S.L., Arganda del Rey, Madrid, Spain) device was mounted as follows: a non-vented central piece was placed on top of the one-day-old growing Actinomycete plate (facing up) and the other plate was placed upside down on top of them (Figure 1). The chamber device has a hole in the central part (without any type of film or filter covering it), allowing the exchange of VOCs between the cultures and avoiding physical contact between the strains or with the compounds that diffuse into the medium. The assembled VOC chamber was sealed with Parafilm® (Bemis, E-Thermo Fisher Scientific, Madrid, Spain). The dual culture was incubated at 28 °C for 5 days. Control VOC chambers were also set with one noninoculated plate and one inoculated plate. Each experiment was made in triplicate.

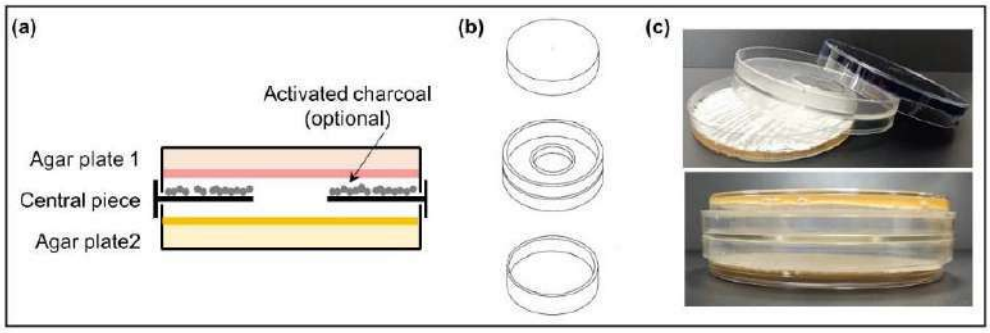

Figure 1. Volatile compound (VOC) chamber. (**a**) Schematic side-view of a VOC chamber; (**b**) representation of each part of the VOC chamber device. The hole in the middle allows the exchange of VOCs between cultures (modified from [25]); (**c**) photographs of an assembled VOC chamber (without charcoal).

2.3. Multiple Coculture of Actinomycetes

Streptomyces sp. CS065a, CS207, CS113, CS149, and CS090a were cultured on YMA or SFM small Petri dishes (diameter of 5 cm) at 28 °C. After 24 h, four opened small plates were placed inside a large Petri dish (diameter of 13,5 cm) and this was sealed with Parafilm® (Bemis, E-Thermo Fisher Scientific, Madrid, Spain). As a control, three noninoculated small plates and the fourth inoculated plate were settled on a large plate. The culture was incubated at 28 °C for 5 days. Each experiment was made in triplicate.

2.4. Antibiotic Production in Co-Culture

Agar diffusion bioassays against *Micrococcus luteus* (Gram-positive bacteria), *Escherichia coli* (Gram-negative bacteria), the yeast *Candida albicans*, and the ascomycete *Escovopsis weberi* were performed to test antibiotic production in cocultures. Fresh cultures or fungal spores of each test microorganism were used as seed cultures to inoculate agar plates of TSA (for bacteria), YMA (for yeast), or SFM (for fungus). A 6 mm agar plug from each Actinomycete plate of the cocultures grown for 5 days in the VOC chambers was placed on top of the bioassay plate. The plates were then incubated at 4 °C for one hour to allow metabolites to diffuse into the surrounding medium. Subsequently, the plates were incubated for 16 h at 30 °C (antifungal tests) or 37 °C (antibacterial tests). Agar plugs from control plates grown in single culture were also used. The diameter of the inhibition zones was measured and compared with the control sample. Each test was performed in triplicate.

2.5. Extraction of Secondary Metabolites Produced in VOC Chambers, Analysis with UPLC, and Dereplication

We then extracted 2.5 g of the actinomycete agar plates grown in coculture using 3 mL of different organic solvents [ethyl acetate, ethyl acetate containing formic acid (1%)

or butanol] and analyzed the extract via reverse phase chromatography in an Acquity UPLC instrument fitted with a BEHC18 column (1.7 µm, 2.1 mm × 100 mm, Waters), with acetonitrile and MQ water + 0.1% trifluoroacetic acid (TFA) as the mobile phase. The PDA detector was set to scan wavelengths between 200 and 600 nm. Samples were eluted with acetonitrile (10%) for 1 min, followed by a linear gradient of acetonitrile (10–100%) for 7 min (flow rate of 0.5 mL/min; column temperature 35 °C). The identity of the metabolites present in these samples was checked via HRMS-based dereplication against MEDINA using an Agilent 1200 Rapid Resolution HPLC coupled with a maXis Bruker qTOF mass spectrometer. The volume injected was 2 µL and a Zorbax SB-C8 column (2.1 × 30 mm, 3.5 µm particle size) was used for separation. The mobile phase consisted of solvent A, 90:10 milliQ water-acetonitrile, and solvent B, milliQ water-acetonitrile, both with 13 mM ammonium formate and 0.01 TFA. Samples were eluted with a 0.3 mL/min flow rate, and the gradient used was 90% to 0% to solvent A/10% to 100% solvent B in 6 min, 0% solvent A/100% solvent B in 2 min, 0% to 90% solvent A/10% to 100% solvent B in 0.1 min, and 90% solvent A/10% solvent B for 1.9 min. The maXis qTOF mass spectrometer was operated in ESI positive mode. Source conditions were 4-kV capillary voltage, end plate offset = 500 V, dry gas (N_2) flow = 11 L/min; dry temperature = 200 °C, and nebulizer (N_2) pressure at 2.8 bars. Transfer line conditions were RF 300 Vpp, isCD energy = 0 eV, hexapole = 60 Vpp, quadrupole ion energy = 5 eV, collision cell energy = 10 eV. The mass spectrometer operated with a mass range of m/z 150–2000 and a spectral acquisition rate of 3 Hz. TFA-Na cluster ions were used for mass calibration of the instrument prior to sample injection. Prerun calibration was via infusion with the same TFA-Na calibrant. The retention time, together with the exact mass (and the derived molecular formula), was used as a criterion to search the internal database from Fundación MEDINA [30] and the Dictionary of Natural Products version 26:2 [31] to identify already known compounds.

2.6. Purification of Rumycins

Thirty VOC chambers were mounted as described in Section 2.2, placing the strains CS149 and CS131 cultured on SFM and incubated at 28 °C for seven days across from each other. The thirty plates in which the CS149 strain was grown were extracted with 600 mL of butanol and subsequently filtered, concentrated under vacuum and resuspended in MQ water. The sample was fractionated through a 10 g Sep-PaK® Vac 35 cc C18 cartridge (Waters) using as mobile phase solvent methanol: MQ water at 5 mL/min and a gradient of 0% to 100% methanol for 55 min.

Purification of the desired fractions was carried out via reverse phase chromatography on an Alliance HPLC chromatographic system (Waters 2695 Separation Module) coupled to a Waters 996 Photodiode Array Detector, using a Sunfire C18 column (10 µm, 10 mm × 280 mm, Waters) and an isocratic mixture 55:45 MQ Water: ACN for rumycin 1 and 20:80 for rumycin 2 at a flow rate of 5 mL/min. The compounds were collected and lyophilized.

2.7. Biosynthetic Gene Cluster Prediction and Sequence Analysis

Biosynthetic gene cluster prediction for secondary metabolite searches and sequence analysis was carried out with the online bioinformatic tool antiSMASH v6 [32]. Rumycin gene clusters were deposited at the Minimum Information about a Biosynthetic Gene Cluster (MIBiG) repository [33] under the accession number BGC0002753.

3. Results and Discussion

3.1. Morphological and Developmental State of Actinomycete strains in VOC Chambers

The morphology of strains growing on R5A, SFM, and YMA agar plates was compared in monoculture versus coculture in VOC chambers. No changes in growth rate were detected and only a slight difference was observed in the timing of the sporulation stage depending on the particular strain and the strain with which it was paired. In addition, exploratory behavior at the edge of the colonies was evaluated as a previous study had revealed the importance of VOCs in this kind of development (Figure S1, Supplementary

data). Fungal VOCs triggered exploratory growth in *Streptomyces venezuelae* colonies, and these "activated" cells could induce this developmental state in other physically separated *Streptomyces* colonies by producing the airborne compound trimethylamine (TMA) [34]. In the present study, exploratory growth was not observed in any of the tested strains. This result could be due to the lack of TMA production by the CS strains or by a different response to this VOC by the Actinomycete strains employed in the present assay. Further work is ongoing trying to clarify this point.

The only exception to the observations was *Streptomyces* sp. CS194. When cultured on SFM medium, this strain was unable to grow in monoculture (control). Only when exposed to VOCs from other CS strains did CS194 reach different levels of development (Figure 2). This fact highlights the importance of VOCs in the communication between strains within a complex community and demonstrates their role as growth-promoting agents. Traditionally, microbial growth and development studies have been mainly focused on pure-culture systems, but now there is increasing evidence that cannot be avoided: microbes live in changing systems of multiple species, and for that reason, the interaction between them should be taken into account to gain a deeper understanding of microbial physiology [35,36]. Several studies have referred to the developmental changes induced by water-diffusible compounds. The siderophore desferrioxamine E enhanced growth and antibiotic activity in several *Streptomyces* species as well as the production of goadsporin, a microcin-like peptide [37,38]. In addition, volatile γ-butyrolactones have been described as quorum sensing molecules that stimulate aerial growth and metabolite production in *Streptomyces* [39,40]. Most of the strains that promoted the growth and development of strain CS194 presented in their genomes BGCs responsible for the synthesis of different types of butyrolactones [41], thus the growth-promoting effect observed in this work could be due to this family of compounds. More experiments need to be performed to elucidate this point.

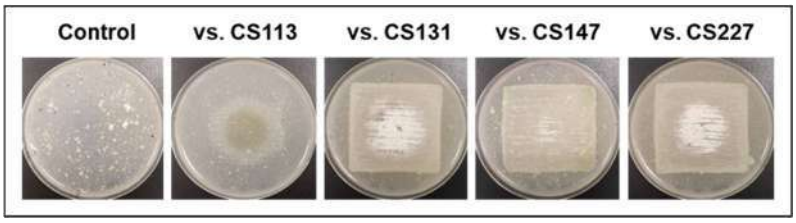

Figure 2. Strain CS194 grown in SFM medium paired with different CS strains in VOC chambers.

3.2. New or Incremental Increases in Antibiotic Production in VOC Chambers

Agar plate bioassays were carried out using samples extracted from the CS strains grown in VOC chambers and inhibition areas were compared to controls (strains grown in monoculture). No positive results were obtained when strains were cultivated on R5A and only strains CS149 and CS194 showed increased antibiotic activity in coculture when grown on SFM medium. On the contrary, the secondary metabolism of many more strains was activated by VOCs emitted by other colonies on YMA. The results obtained could be summarized as follows (complete results are shown in Supplementary Materials Tables S1–S13; Data concerning the characterization of compounds by LC-MS are available in Supplementary Material file: LC-MS dereplication):

- CS014: An increased antibiotic activity against *M. luteus* was detected in coculture with CS057, CS081a, CS090a, CS131, and CS149 strains grown on YMA plates (Figure 3a,b). Comparative UPLC analysis revealed the activation of collismycin production in the presence of VOCs from the strains mentioned above. Furthermore, increased production of granaticin C was observed (Figure 3c).
- CS081a: This strain only demonstrated anti-*M. luteus* activity when grown on YMA medium in VOC chambers cultured against CS014 strain (Figure 3b). The chromatographic analysis of samples extracted with ethyl acetate exposed the biosynthetic induction of the cosmomycin anthracycline antibiotic family by CS081a under the effect

of CS014 VOCs (Figure 3d). In this case, the signaling caused by volatiles emitted by the strains inside the chamber was bidirectional as VOCs from CS014 impacted the secondary metabolism of the CS081a strain and vice versa (see the previous paragraph).

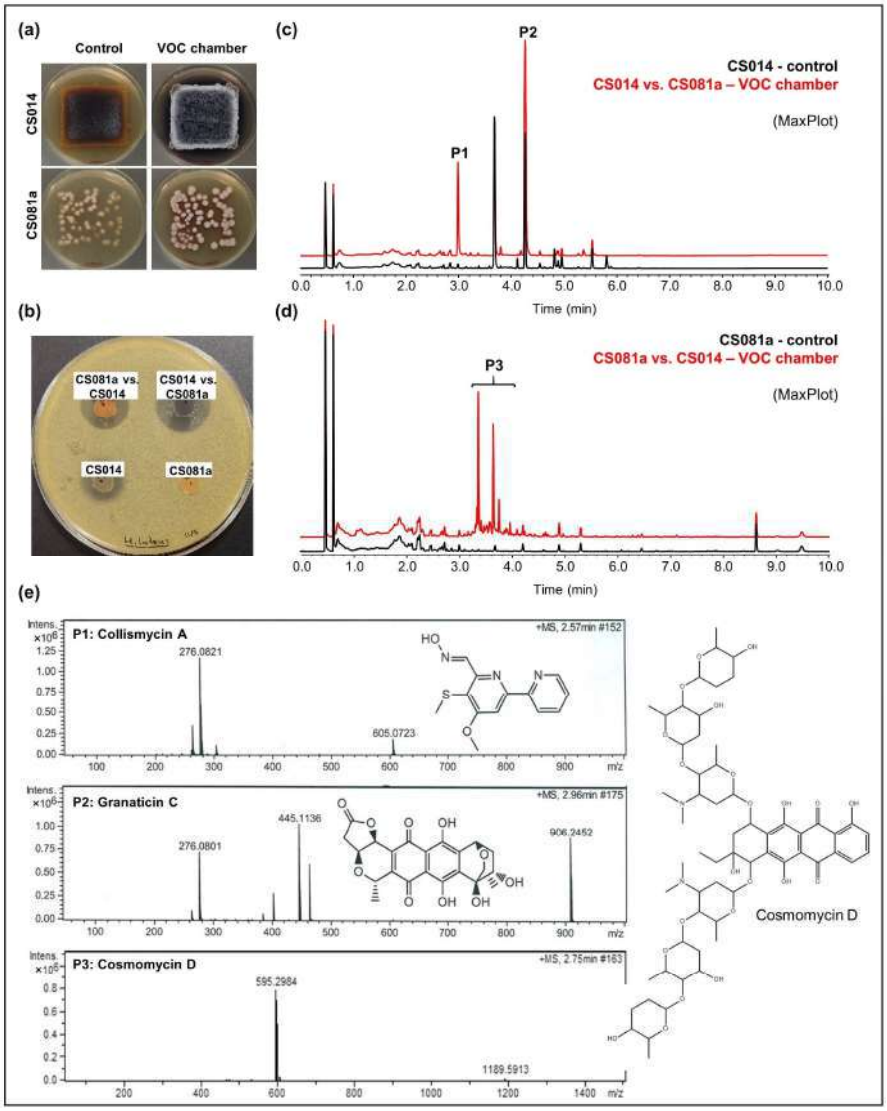

Figure 3. Overview of the results obtained for the strains CS014 and CS081a grown on YMA medium. (**a**) Upper-view photographs of YMA plates where the strains were grown alone (control plates) or co-cultured in a VOC chamber; (**b**) bioassay plate against *Micrococcus luteus*; (**c**,**d**) UPLC chromatograms (MaxPlot) of samples extracted with ethyl acetate. Peaks observed only (or in a higher amount) in co-culture were highlighted; (**e**) HRMS spectra and chemical structures of the related compounds.

- CS057: The strain growing on YMA agar plates in a VOC chamber together with CS081a demonstrated stronger bioactivity against *M. luteus* and *E. weberi*. UPLC analysis revealed an increased production of the related compounds cycloheximide and actiphenol by CS057 exposed to VOCs from CS081a (Figure 4a,c,d).
- CS090a: Antifungal compounds active against *E. weberi* were only produced by the CS090a strain when it was grown on YMA under the effect of VOCs originating from CS057 and CS081a. Chromatographic analysis found maltophilin and alteramide activation of the production (Figure 4b,e,f).

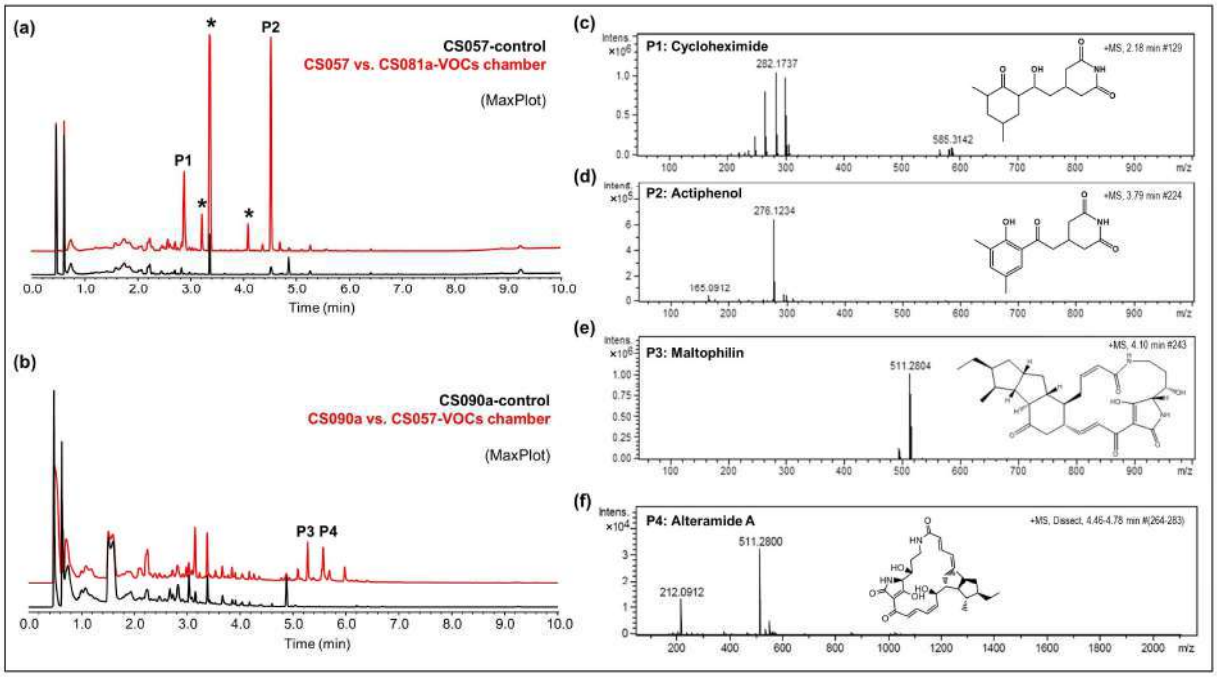

Figure 4. Activation of secondary metabolism in CS057 and CS090a strains. (**a**) Comparative UPLC analysis of samples of CS057 (control plate, black) and CS057 exposed to VOCs produced by CS081a (red). Asterisks indicate intermediates of actiphenol biosynthesis; cycloheximide (P1); actiphenol (P2). (**b**) Comparative UPLC analysis of samples of CS090a (control plate, black) and CS090a exposed to VOCs produced by CS057 (red). Maltophilin (P3) and alteramide A (P4); (**c**) HRMS spectrum of cycloheximide, (**d**) HRMS spectrum of actiphenol, (**e**) HRMS spectrum of maltophilin; (**f**) HRMS spectrum of alteramide A.

- CS149: No antibiotic activity against the tested microorganisms was detected when CS149 was grown in monoculture. However, when paired with CS081a or CS131 in VOC chambers, potent bioactivity against *M. luteus* was observed. Chromatographic analysis highlighted the activation of the production of two different compounds that were not present in samples extracted from monocultures of CS149 (Figure 5a). These compounds were identified by dereplication as the anti-MRSA cyclic lipopeptides rumycin 1 and rumycin 2 (Figure 5b–d). In a step-forward study to confirm that the observed alteration of the CS149 secondary metabolism was due to the VOCs emitted by CS131, a VOC chamber with 2 g of activated charcoal was assembled and then the samples extracted with ethyl acetate from SFM CS149-CS131 dual cultures were analyzed by UPLC. No rumycins could be detected (Figure 5a) indicating that VOCs from CS131 were adsorbed by the activated charcoal, and thus could not exert their inductive effect on CS149 biosynthetic machinery. On the other hand, biosynthesis of rumycins by *Streptomyces* sp. CS149 does not depend strictly on the growth medium of the strain; this has been verified in both SFM and R5A. Otherwise, the VOCs that induced the activation of the secondary metabolism of *Streptomyces* sp. CS149 were only produced by CS081a or CS131 if grown on SFM since rumycins could not be detected when the VOC-emitting strain was cultured on R5A or YMA. We purified 3.6 mg of rumycin 1 and 2.8 mg of rumycin 2 from thirty plates (total volume of 600 mL) of SFM (dual cultures between CS149 and CS131 strains in VOC chambers). Pure compounds were used for the testing of bioactivity via agar diffusion bioassay. They demonstrated potent antibacterial activity against *M. luteus*, even stronger than the commonly used antibiotic apramycin, but no activity against *E. coli*, *C. albicans*, and *E. weberi* (Figure 5e).

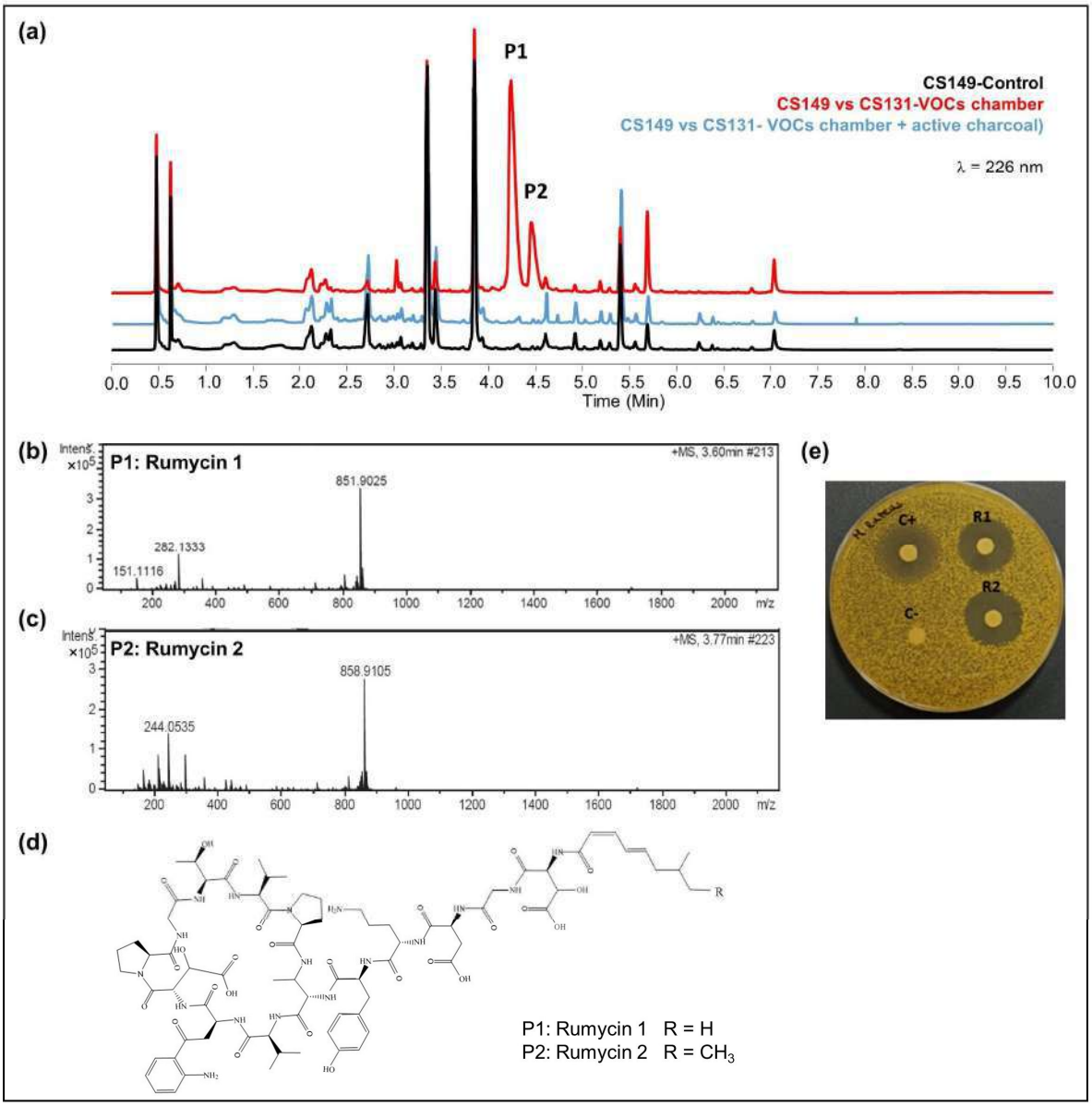

Figure 5. Activation of the synthesis of rumycins by the effects of VOCs from *Streptomyces* sp. CS131 on CS149. (**a**) Comparative UPLC profile where the peaks containing rumycin 1 and rumycin 2 have been numbered as P1 and P2, respectively; (**b**) HRMS spectrum of rumycin 1; (**c**) HRMS spectrum of rumycin 2. (**d**) Chemical structure of rumycins. (**e**) Bioassay of purified rumycins against *M. luteus*. (C^+): apramycin (200 mg/mL), (C^-): methanol, (R1): rumycin 1 (10 mg/mL), (R2): rumycin 2 (10 mg/mL).

- CS194: When cultivated on SFM, the strain CS194 exposed to VOCs emitted by strains CS014, CS057, CS081a, or CS227, presented antibiotic activity versus *M. luteus*. As mentioned previously, this strain was not able to grow on SFM in monoculture, thus we could not state if the production of the antibiotic/s was due to a signaling effect of VOCs on growth promotion or by the induction of the CS194 secondary metabolism (or both). In any case, the antibacterial activity of CS194 could be explained by the production of bioactive piperazinediones, detected by dereplication in samples of CS194 (Figure S2, Supplementary data).
- CS207: The antibacterial (against *M. luteus* and *E. coli*) and antifungal activities of the strain CS207 were higher when cultivated on YMA in VOC chambers together with strains CS014, CS057, CS081a, or CS227. Unfortunately, the comparative UPLC analysis

did not retrieve any differential peak that could explain the observed antibiotic activity, probably due to the limitation of the analytics based on UV absorbance measurements.

The close relationship between leaf cutter ants and Actinomycetes has been extensively studied for decades [22,23,42]. Although the true symbiont of the *Attini* ants has been identified as *Pseudonocardia* sp. [43], the role of the Streptomycete population isolated from the surface of those insects has not been fully elucidated. Batey and colleagues reviewed the involvement of these Streptomycetes in protecting the nests from pests using the production of specialized antimicrobial compounds [23]. Thus, symbiotic, mutualistic and antagonistic behaviors take place in the confined spaces delimited by the subterranean nest chambers, in such a way that a controlled network of interspecific communication signals plays a key role in maintaining the stability of the community.

In this work, we have demonstrated the potential of the CS strains as antibiotic producers but, more importantly, their ability to modulate the biosynthetic machinery of other related species. VOCs emitted by these strains provoke the overproduction of compounds with diverse chemical structures known for their remarkable antibiotic activity, as is the case of the benzoisochromanequinone polyketide granaticins [44], the polycyclic tetramate macrolactam alteramides [45], and the glutarimide-containing polyketide family of cycloheximide and actiphenol [46,47], synthesized by *Streptomyces* sp. CS014, CS090a, and CS057, respectively.

Furthermore, the application of these VOCs in activating silent biosynthetic gene clusters that could lead to the discovery of new compounds with potential biomedical uses is very promising and could be implemented as a routine technique to carry out during drug screening research programs. Interspecific communication between different Actinomycetes mediated by VOCs induced the production of several bioactive compounds that were not biosynthesized when the strains were grown in monoculture. Within the VOC chambers, we observed the induction of the biosynthesis of collismycins (cytotoxic and antibiotic 2,2′-bipyridyl class of compounds [48]), cosmomycins (glycosylated anthracyclines with antibiotic properties [49]), maltophilins (macrolactams with antifungal activity [50]), alteramides, and rumycins (cyclic lipopeptides with strong antibacterial properties, proposed as a treatment against methicillin-resistant *Staphylococcus aureus* infections [51]). The potent bioactivity of rumycins against *M. luteus* observed during this work makes them good candidates for further research on their biosynthesis.

Notably, the same biosynthetic machinery was activated by VOCs from different strains (e.g., collismycins and granaticins were produced by *Streptomyces* sp. CS014 when exposed to VOCs from five different strains, CS057, CS081a, CS090a, CS131, and CS149). This fact could indicate a common mechanism among Actinomycetes to modulate the secondary metabolism of other related bacteria. One possible explanation could be the production of the same VOC by different strains that triggered the expression of one specific BGC. On the contrary, another plausible mechanism could be the manifestation of the same response of the induced metabolic machinery to different VOCs.

3.3. Effect of VOCs in Multiple Co-Culture

There were some strains in which we could not detect any modification in their capacity to produce antibiotic compounds after being exposed to VOCs from other Actinomycetes in VOC chambers. With those strains, we performed a multiple co-culture approach where the strains were under the effect of VOCs from other three different strains to mimic the complex environment found inside the nests inhabited by the leaf-cutter ants (complete results were shown in Supplementary Materials Tables S14–S18). Applying this approach, we were able to detect higher antibacterial activity against *M. luteus* and *E. coli* in *Streptomyces* sp. CS065a when exposed to VOCs from different combinations of CS strains (Figure 6a). The comparative UPLC profile between samples of *Streptomyces* sp. CS065a grown in monoculture or in multiple cocultures with CS113, CS147, and CS207 (co-culture 1; 1CC); CS090a, CS147, and CS207 (co-culture 2; 2CC; Figure 6b); or CS090a, CS113 and CS207 (co-culture 3; 3CC) revealed activation of the production of several compounds

of the alteramide and chromomycin families (Figure 6c,d). The antimicrobial activity against Gram-positive bacteria could be explained by the action of these two groups of compounds [45,52]. Therefore, the observed bioactivity against *E. coli* might in addition be due to the production of one or more compounds that could not be detected under our experimental techniques, since alteramides and chromomycins have not been described as anti-Gram-negative agents individually.

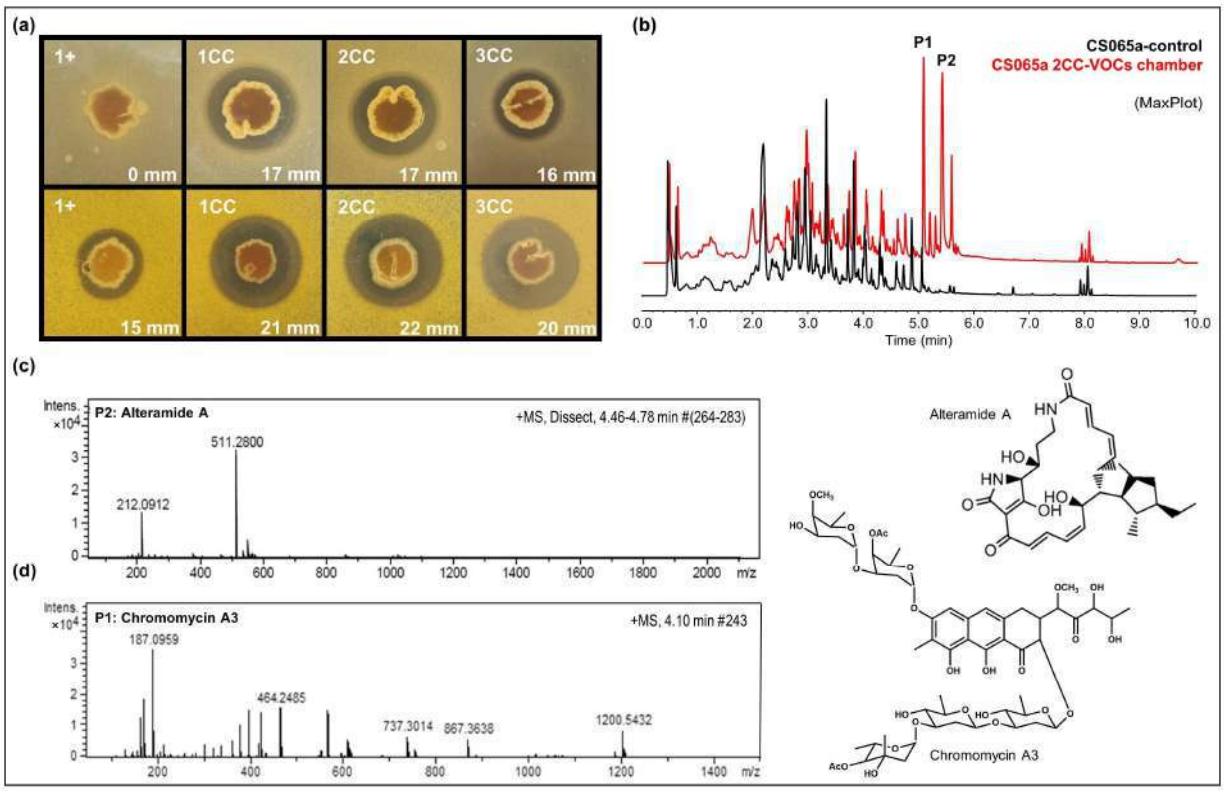

Figure 6. Results of multiple co-culture of CS065a on SFM. (**a**) Bioassay against *E. coli* (first row) and *M. luteus* (second row). 1^{+}: *Streptomyces* CS065; 1CC: co-culture CS065a, CS113, CS147, and CS207; 2CC: co-culture CS065a, CS090a, CS147 and CS207; 3CC: coculture CS065a, CS090a, CS113 and CS207. (**b**) Comparative UPLC analysis (MaxPlot) of samples of CS065a in monoculture (black) versus in co-culture with CS090a, CS147, and 207 (red). Differential peaks are marked with an asterisk. (**c**) HRMS spectrum of alteramide A. (**d**) HRMS spectrum of chromomycin A3.

Coculture has been successfully applied in new drug screening programs because it is capable of mimicking interaction between naturally occurring microbial communities. By co-culturing different bacterial and fungal species, the induction of many compounds (e.g., aminoglycosides, terpenes, polyketides, or alkaloids) has been reported [53]. Most of these studies were based on mixed fermentation or solid medium co-cultures, so it is impossible to determine whether the observed metabolic changes are due to water-diffusible or volatile compounds. We show the importance of VOCs in the metabolic modulation of related species and point out the idea that more than one VOC producer may be needed to obtain the desired results. The biosynthesis of alteramides and chromomycins by *Streptomyces* sp. CS065a only occurred when the strain was co-cultured with three other strains, as we were unable to detect those compounds in samples from monoculture or dual-culture using VOC chambers pairing the same strains.

3.4. Identification of the Rumycin Biosynthetic Gene Cluster

Among the metabolites whose biosynthetic pathways were activated during this work, only rumycins were not linked to a previously described BGC. Therefore, bioinformatic analysis was carried out to identify the rumycin (*rmc*) gene cluster within the genomic

sequence of *Streptomyces* sp. CS149. AntiSMASH v6 predicted the presence of 31 BGCs in the genomic DNA of *Streptomyces* sp. CS149 [41]. Based on the chemical structure of rumycins (cyclic lipopeptides made up of 14 amino acid residues), cluster 17 was identified as the unique candidate for the BGC responsible for the synthesis of rumycins. The *rmc* BGC was classified as an 84 Kb non-ribosomal peptide (NRP) cluster type with a 66% similarity to the cadaside BGC (Figure 7). BLASTp analysis of each *rmc* gene product revealed the presence of genes involved in the synthesis of the NRP chain, transport, regulation, and synthesis of nonproteinogenic amino acids and the acyl chain. A detailed description of the predicted functions of the *rmc* genes is summarized in Table S2 (Supplementary Materials Table S19).

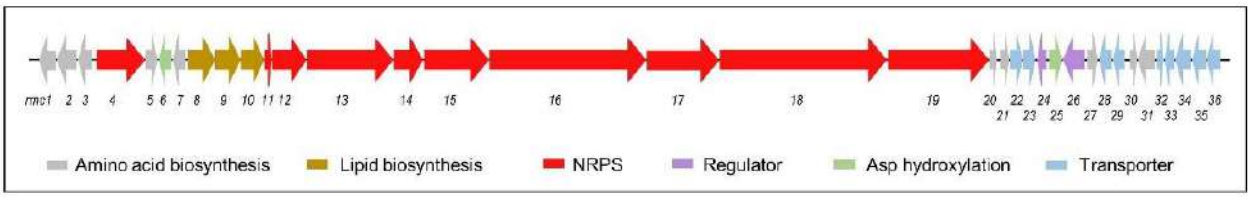

Figure 7. Rumycin (*rmc*) biosynthetic gene cluster.

4. Conclusions

In complex microbial ecosystems, communication between individuals is a crucial factor for the survival and health of the community. Inter- and intra-kingdom signaling plays a key role in the spatial and temporal coordination of cellular developmental processes, contributing to the detection of nutritional stress or activating competitive behaviors through antibiosis [54]. Among the wide array of metabolites produced by microorganisms, the chemical properties of VOCs make them the perfect form of communication in an environment full of air gaps such as soil [55]. In this work, VOC chambers have been successfully applied to study the effect of volatiles (separately from water-diffusible compounds) on the secondary metabolism of related species. The production of bioactive compounds with different chemical natures and target microorganisms has been improved by the signaling effect of VOCs emitted by nearby bacterial strains. In addition to its role in antibiosis, a role in modulating the behavior of the bacterial community to better combat pests could be attributed to the actinomycete microbiome of the leaf-cutting ants. As far as we are concerned, this is the first time the VOC-induced production of bioactive compounds by Actinomycetes has been described, pointing out the potential of volatile compounds as a useful tool for drug discovery.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells11213510/s1, LC-MS dereplication: Data corresponding to the identification of compounds whose production was activated in this work. Figure S1: Complete panel of dual cultures installed in VOC chambers pairing *Streptomyces* sp. CS057 with the rest of the CS strains. Top and bottom views of the YMA agar plates on day 5 of the co-culture. Figure S2: Dereplication analysis of a sample of the CS194 strain grown in SFM medium under the effect of VOCs emitted by CS227. Tables S1–S13: Overview of the complete results obtained in dual co-cultures using VOC chambers. Tables S14–S18: Overview of the complete results obtained in multiple cocultures using VOC chambers. Table S19. Predicted function of *rmc* genes.

Author Contributions: Conceptualization, M.G.M. and C.O.; methodology, M.G.M. and C.O.; software, M.G.M. and L.C.; validation, L.C., M.G.M. and C.O.; formal analysis, L.C., M.G.M. and C.O.; investigation, L.C., C.M., C.O. and M.G.M.; resources, C.M., J.A.S. and C.O.; data curation, L.C. and M.G.M.; writing—original draft preparation, M.G.M. and L.C.; writing—review and editing, L.C., C.M., J.A.S., C.O. and M.G.M.; visualization, L.C., C.O. and M.G.M.; supervision, M.G.M. and C.O.; project administration, C.M., J.A.S. and C.O.; funding acquisition, C.M., J.A.S. and C.O. All authors have read and agreed to the published version of the manuscript.

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Supplementary data

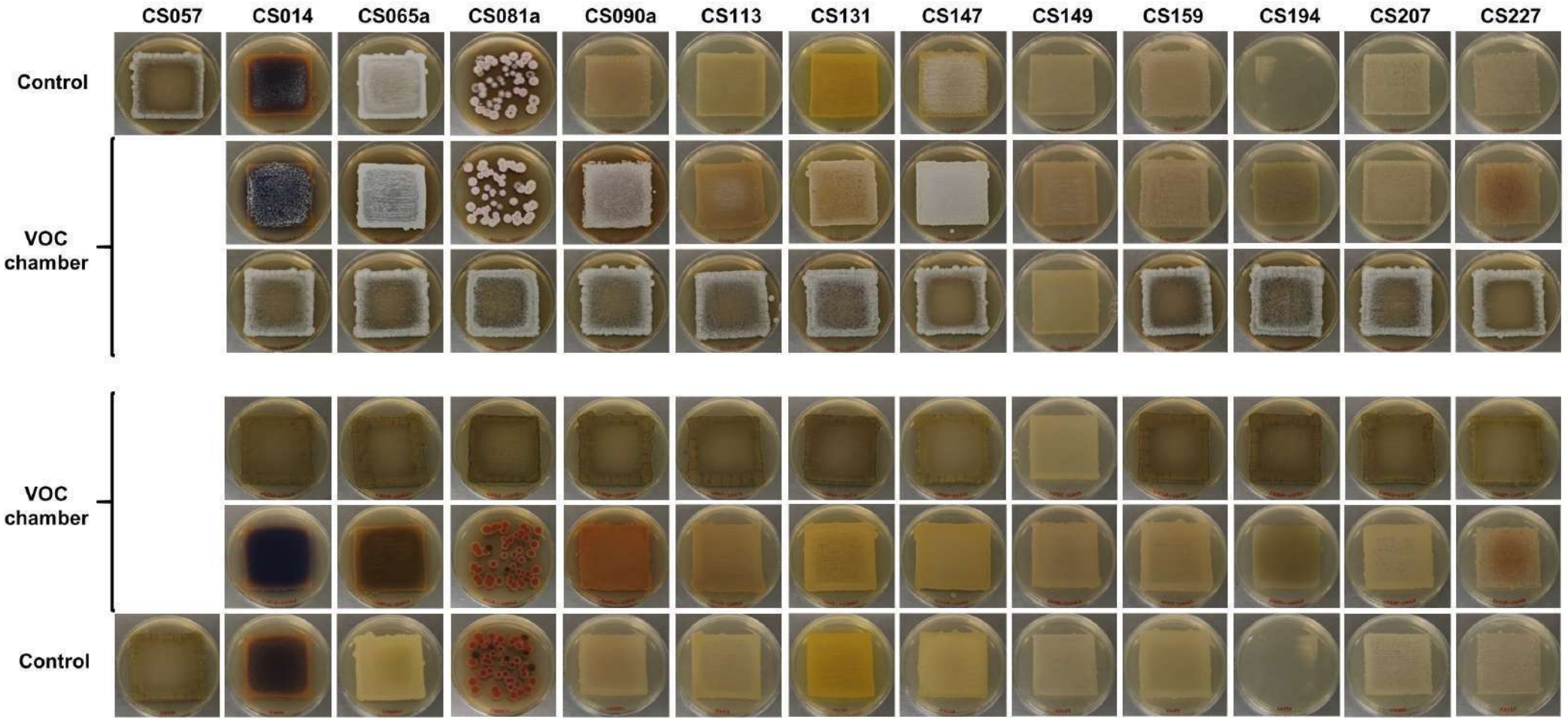
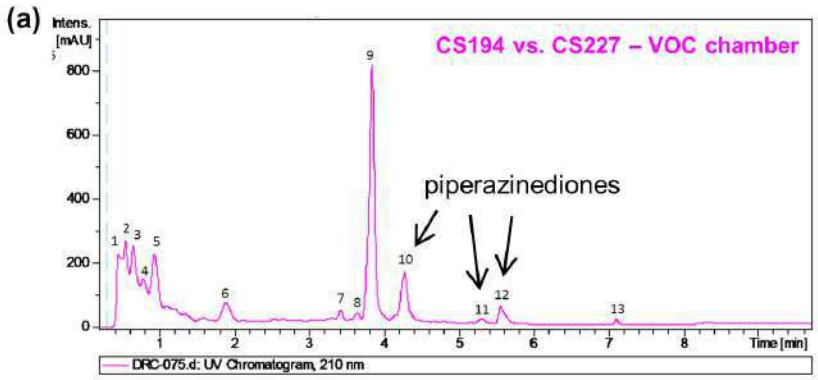
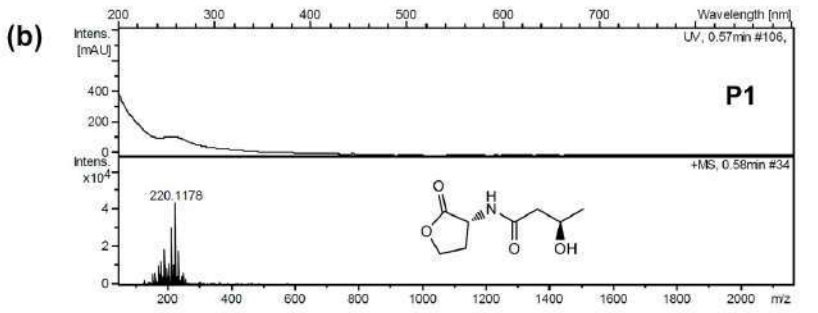
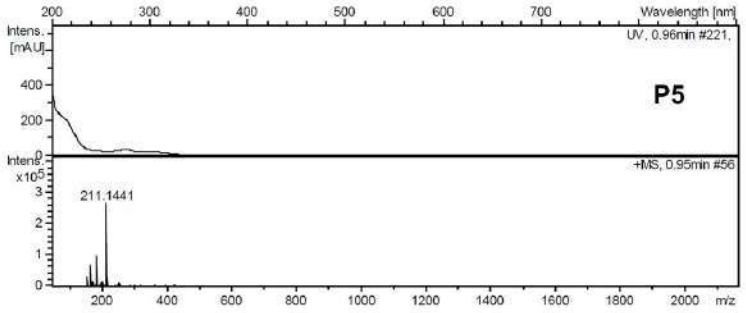


Figure S1. Complete panel of dual cultures installed in VOC chambers confronting *Streptomyces* sp. CS057 with the rest of the CS strains. Top and bottom views of the YMA agar plates on day 5 of the coculture.

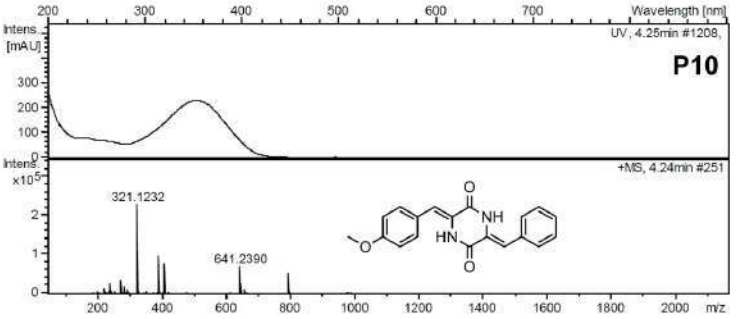




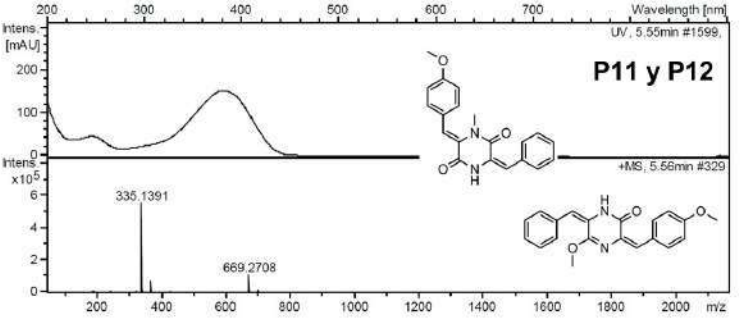
Fórmula molecular sugerida: $C_8H_{13}NO_4$. La única coincidencia que aparece en el DPN para esta fórmula corresponde a **N-(3-Hydroxybutanoyl)homoserine lactone**.



Fórmula molecular sugerida: $C_{14}H_{16}N_2O_2$. **Cyclo(Leu-Pro)**.



Fórmula molecular sugerida: $C_{19}H_{16}N_2O_3$. Única coincidencia en el DPN corresponde a la piperazinediona **XR 334**.



Fórmula molecular sugerida: $C_{20}H_{18}N_2O_3$. Dos coincidencias en el DPN, siendo las dos piperazinodionas: **XR 330** y **4'-Methoxyneihumicin**

Figure S2. Dereplication analysis of a sample of the strain CS194 grown in SFM medium under the effect of VOCs emitted by CS227. (a) UPLC chromatogram at 210 nm; (b) UV-absorption and HRSM spectra of identified peaks.

Table S1. Overview of the dual co-culture against *Streptomyces* sp. CS014.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS014
CS057	SFM	No effects observed
	YMA	Increased bioactivity against *M. luteus* (collismycin and granaticin)[1]
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	Increased bioactivity against *M. luteus* (collismycin and granaticin)[1]
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	Increased bioactivity against *M. luteus* (collismycin and granaticin)[1]
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	Increased bioactivity against *M. luteus* (collismycin and granaticin)[1]
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	Increased bioactivity against *M. luteus* (collismycin and granaticin)[1]
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

[1] Collismycin was only produced in coculture. Higher granaticin production in coculture compared to monoculture.

Table S2. Overview of the dual co-culture against *Streptomyces* sp. CS057.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS057
CS014	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	Increased bioactivity against *M. luteus* and *E. weberi* (cycloheximide and actiphenol)[1]
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	Slight increased bioactivity against *M. luteus* and *E. weberi* (cycloheximide and actiphenol)[1]
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

[1] Only traces of cycloheximde and actiphenol were detected in monoculture.

Table S3. Overview of the dual co-culture against *Streptomyces* sp. CS065a.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS065a
CS014	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

Table S4. Overview of the dual co-culture against *Streptomyces* sp. CS081a.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS081a
CS014	SFM	No effects observed
	YMA	Increased bioactivity against *M. luteus* (cosmomycins)[1]
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	Slight increased bioactivity against *M. luteus* (cosmomycins)[1]
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

[1] Cosmomycins were only produced in coculture.

Table S5. Overview of the dual co-culture against *Streptomyces* sp. CS090a.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS090a
CS014	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	Bioactivity against *E. weberi* (maltophilins and alteramides)[1]
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	Bioactivity against *E. weberi* (maltophilins and alteramides)[1]
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

[1] Maltophilins and alteramides were only produced in coculture.

Table S6. Overview of the dual co-culture against *Streptomyces* sp. CS113.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS113
CS014	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

Table S7. Overview of the dual co-culture against *Streptomyces* sp. CS131.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS131
CS014	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

Table S8. Overview of the dual co-culture against *Streptomyces* sp. CS147.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS147
CS014	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

Table S9. Overview of the dual co-culture against *Streptomyces* sp. CS149.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS149
CS014	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	Bioactivity against *M. luteus* (rumycins)[1]
	YMA	No effects observed
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	Bioactivity against *M. luteus* (rumycins)[1]
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

[1] Rumycins were only produced in coculture.

Table S10. Overview of the dual co-culture against *Streptomyces* sp. CS159.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS159
CS014	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

Table S11. Overview of the dual co-culture against *Streptomyces* sp. CS194.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS194
CS014	SFM	Bioactivity against *M. luteus* (piperazinediones?)[1]
	YMA	No effects observed
	R5A	No effects observed
CS057	SFM	Bioactivity against *M. luteus* (piperazinediones?)[1]
	YMA	No effects observed
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	Bioactivity against *M. luteus* (piperazinediones?)[1]
	YMA	No effects observed
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	Difference in the timing of the sporulation
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	Difference in the timing of the sporulation
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	Difference in the timing of the sporulation
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	Difference in the timing of the sporulation and bioactivity against *M. luteus* (piperazinediones?)[1]
	YMA	No effects observed
	R5A	No effects observed

[1] It was not possible to make a comparative study of the metabolites produced by CS194 grown in monoculture vs. coculture because the strain did not grow in monoculture. Bioactive piperazinediones were detected in coculture samples and their involvement in the observed bioactivity should be taken as a hypothesis.

Table S12. Overview of the dual co-culture against *Streptomyces* sp. CS207.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS207
CS014	SFM	No effects observed
	YMA	Increased bioactivity against *E. coli* and *M. luteus*
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	Increased bioactivity against *E. coli* and *M. luteus*
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	Increased bioactivity against *E. coli* and *M. luteus*
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS227	SFM	No effects observed
	YMA	Increased bioactivity against *E. coli* and *M. luteus*
	R5A	No effects observed

Table S13. Overview of the dual co-culture against *Streptomyces* sp. CS227.

Strain	Growth medium	Outcomes in *Streptomyces* sp. CS227
CS014	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS057	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS065a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS081a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS090a	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS113	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS131	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS147	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS149	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS159	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS194	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed
CS207	SFM	No effects observed
	YMA	No effects observed
	R5A	No effects observed

Table S14. Overview of the multiple co-culture against *Streptomyces* sp. CS065a.

Strains	Growth medium	Outcomes in *Streptomyces* sp. CS065a
CS147, CS207, CS113	SFM	Increased bioactivity against *M. luteus* (alteramide, chromomycin) and bioactivity against *E. coli*
	YMA	Increased bioactivity against *M. luteus* (alteramide, chromomycin) and *E. coli*
CS147, CS207, CS090a	SFM	Increased bioactivity against *M. luteus* (alteramide, chromomycin) and bioactivity against *E. coli*
	YMA	Increased bioactivity against *M. luteus* (alteramide, chromomycin) and *E. coli*
CS147, CS090a, CS113	SFM	No effects observed
	YMA	Increased bioactivity against *M. luteus* (alteramide, chromomycin) and showed bioactivity against *E. coli*
CS090a, CS207, CS113	SFM	Increased bioactivity against *M. luteus* (alteramide, chromomycin) and showed bioactivity against *E. coli*
	YMA	Increased bioactivity against *M. luteus* (alteramide, chromomycin) and *E. coli*

Table S15. Overview of the multiple co-culture against *Streptomyces* sp. CS090a.

Strains	Growth medium	Outcomes in *Streptomyces* sp. CS090a
CS147, CS207, CS113	SFM	No effects observed
	YMA	No effects observed
CS147, CS207, CS065a	SFM	No effects observed
	YMA	No effects observed
CS147, CS065a, CS113	SFM	No effects observed
	YMA	No effects observed
CS065a,CS207, CS113	SFM	No effects observed
	YMA	No effects observed

Table S16. Overview of the multiple co-culture against *Streptomyces* sp. CS113.

Strains	Growth medium	Outcomes in *Streptomyces* sp. CS113
CS147, CS207, CS090a	SFM	No effects observed
	YMA	No effects observed
CS147, CS207, CS065a	SFM	No effects observed
	YMA	No effects observed
CS147, CS065a, CS090a	SFM	No effects observed
	YMA	No effects observed
CS065a,CS207, CS090a	SFM	No effects observed
	YMA	No effects observed

Table S17. Overview of the multiple co-culture against *Streptomyces* sp. CS147.

Strains	Growth medium	Outcomes in *Streptomyces* sp. CS147
CS113, CS207, CS090a	SFM	No effects observed
	YMA	No effects observed
CS113, CS207, CS065a	SFM	No effects observed
	YMA	No effects observed
CS113, CS065a, CS090a	SFM	No effects observed
	YMA	No effects observed
CS065a,CS207, CS090a	SFM	No effects observed
	YMA	No effects observed

Table S18. Overview of the multiple co-culture against *Streptomyces* sp. CS207.

Strains	Growth medium	Outcomes in *Streptomyces* sp. CS207
CS113, CS147, CS090a	SFM	No effects observed
	YMA	No effects observed
CS113, CS147, CS065a	SFM	No effects observed
	YMA	No effects observed
CS113, CS065a, CS090a	SFM	No effects observed
	YMA	No effects observed
CS065a,CS147, CS090a	SFM	No effects observed
	YMA	No effects observed

Table S19. Predicted function of *rmc* genes.

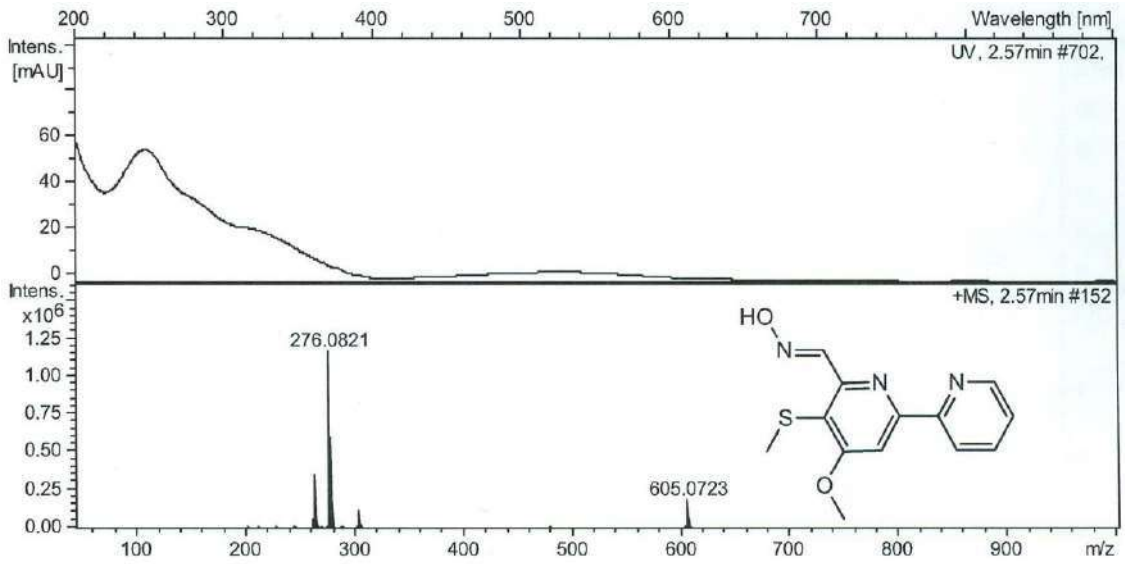
Gene	Function	Closest homologue	
rmc1	diaminobutyric acid synthase C	*Streptomyces viridochromogenes*	AEG64688.1
rmc2	argininosuccinate lyase	*Streptomyces globisporus*	MCC8479050.1
rmc3	pyridoxal-phosphate dependent enzyme (cysteine synthetase)	*Streptomyces globisporus*	MCC8479049.1
rmc4	NRPS	*Streptomyces* sp. CAI-121	NUV71303.1
rmc5	Kinase	*Streptomyces badius*	WP_199887694.1
rmc6	TauD/TfdA family dioxygenase	Multispecies - *Streptomyces*	WP_069738900.1
rmc7	tryptophan 2,3-dioxygenase family protein	Multispecies - *Streptomyces*	WP_079149654.1
rmc8	fatty acyl-AMP ligase	*Streptomyces globisporus*	MCC8479045.1
rmc9	acyl-CoA dehydrogenase family protein	*Streptomyces globisporus*	MCC8479044.1
rmc10	acyl-CoA dehydrogenase	*Streptomyces globisporus*	MCC8479043.1
rmc11	acyl carrier protein	*Streptomyces globisporus*	MCC8479042.1
rmc12	NRPS	*Streptomyces badius*	WP_199887692.1
rmc13	NRPS	*Streptomyces globisporus*	MCC8482945.1
rmc14	NRPS	*Streptomyces globisporus*	MCC8482950.1
rmc15	NRPS	*Streptomyces globisporus*	MCC8482893.1
rmc16	NRPS	*Streptomyces globisporus*	MCC8482894.1
rmc17	NRPS	*Streptomyces globisporus*	MCC8482950.1
rmc18	NRPS	*Streptomyces globisporus*	MCC8482386.1
rmc19	NRPS	*Streptomyces globisporus*	MCC8482387.1
rmc20	MbtH family protein	*Streptomyces badius*	WP_199889602.1
rmc21	DedA family protein	*Streptomyces parvus*	WP_148904607.1
rmc22	ABC transporter ATP-binding protein	*Streptomyces parvus*	WP_148904605.1
rmc23	ABC transporter permease subunit	*Streptomyces* sp. CAI 127	WP_175515771.1
rmc24	response regulator transcription factor (LuxR)	Multispecies - *Streptomyces*	WP_176735228.1
rmc25	TauD/TfdA family dioxygenase	Multispecies - *Streptomyces*	WP_069737015.1
rmc26	Signal transduction histidine kinase	*Streptomyces* sp. CAI 127	NUW01750.1
rmc27	alpha/beta hydrolase	*Streptomyces* sp. CAI 127	WP_175515773.1
rmc28	ABC transporter permease	*Streptomyces* sp. CAI 127	WP_175515774.1
rmc29	ATP-binding cassette domain-containing protein	*Streptomyces* sp. CAI 127	WP_175515775.1
rmc30	SCO5389 family protein	*Streptomyces* sp. CB02366	WP_073763754.1
rmc31	LLM class flavin-dependent oxidoreductase	*Streptomyces* sp. CAI 127	WP_175515776.1
rmc32	ATP/GTP-binding protein	*Streptomyces globisporus*	WP_118906321.1
rmc33	ABC transporter permease subunit	*Streptomyces globisporus*	WP_118906320.1
rmc34	ATP-binding cassette domain-containing protein	*Streptomyces* sp. SID4931	MYV62918.1
rmc35	ABC transporter permease subunit	*Streptomyces* sp. SID14515	WP_164612903.1
rmc36	ABC transporter ATP-binding protein	*Streptomyces filamentosus* NRRL 15998	EFE77641.1

LC-MS dereplication.

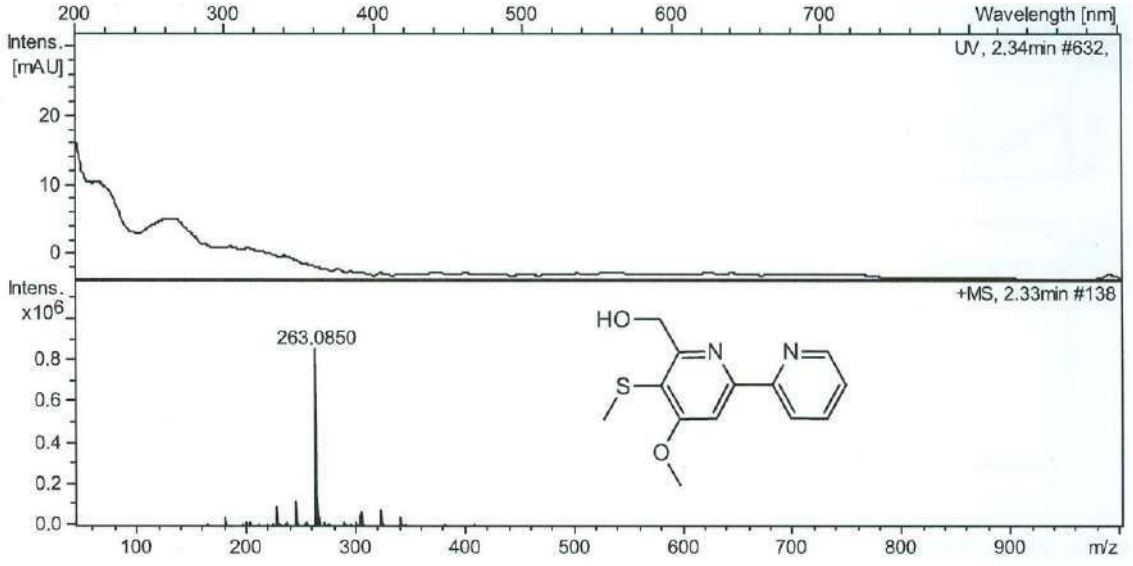
Section 3.2. New or incremented antibiotic production in VOC chambers

CS014:

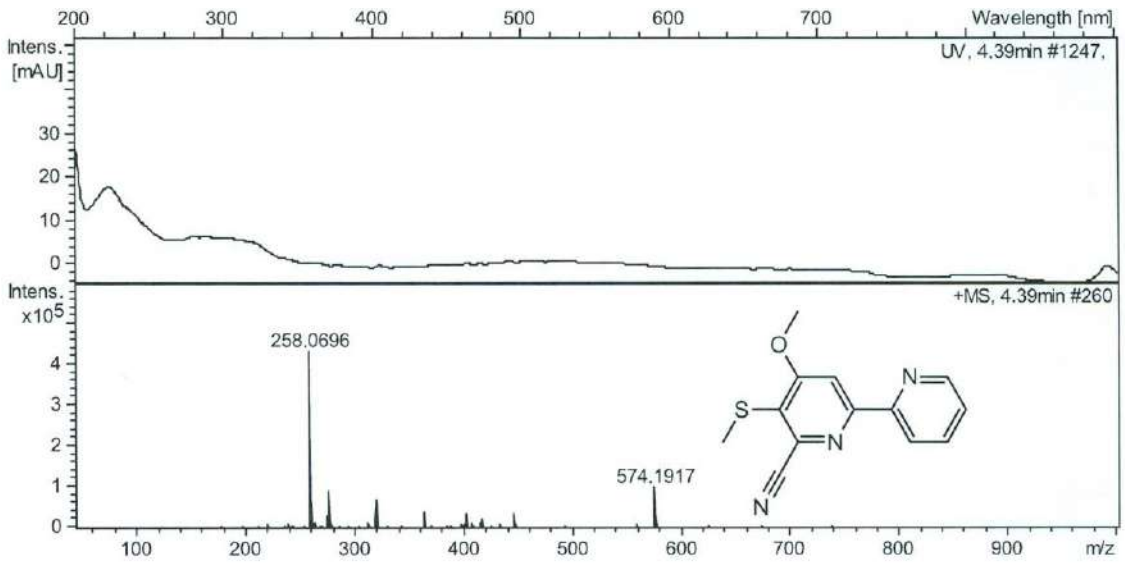
Collismycin A-B, $C_{13}H_{13}N_3O_2S$:



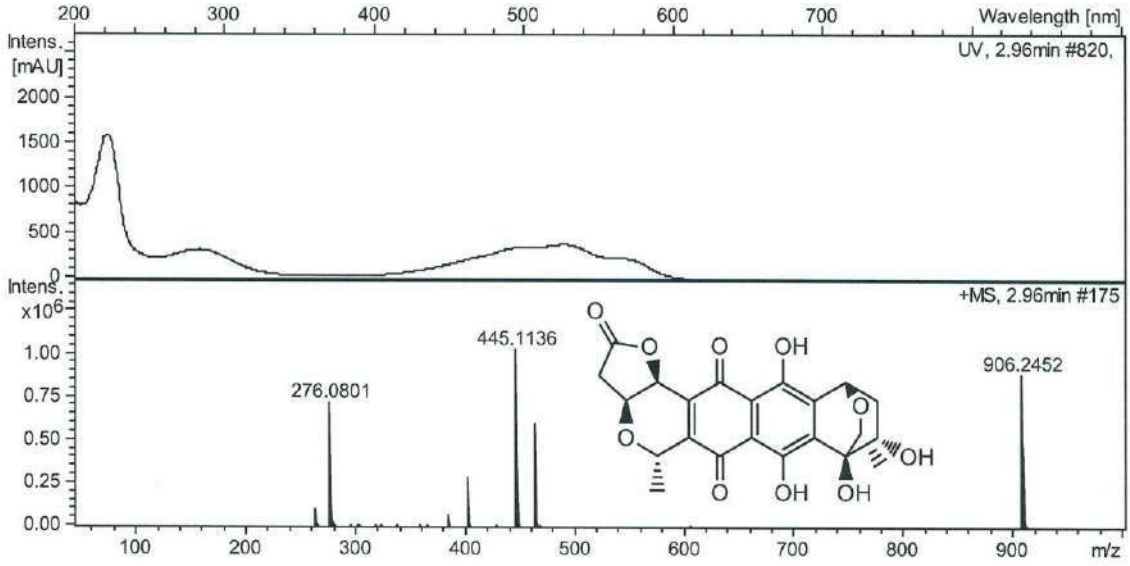
Collismycin C, $C_{13}H_{14}N_2O_2S$:



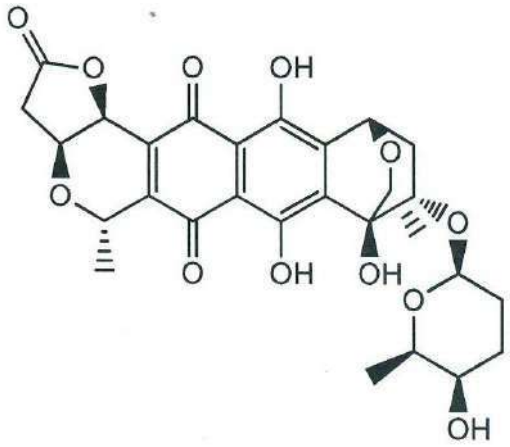
Collismycin D, $C_{13}H_{11}N_3OS$:

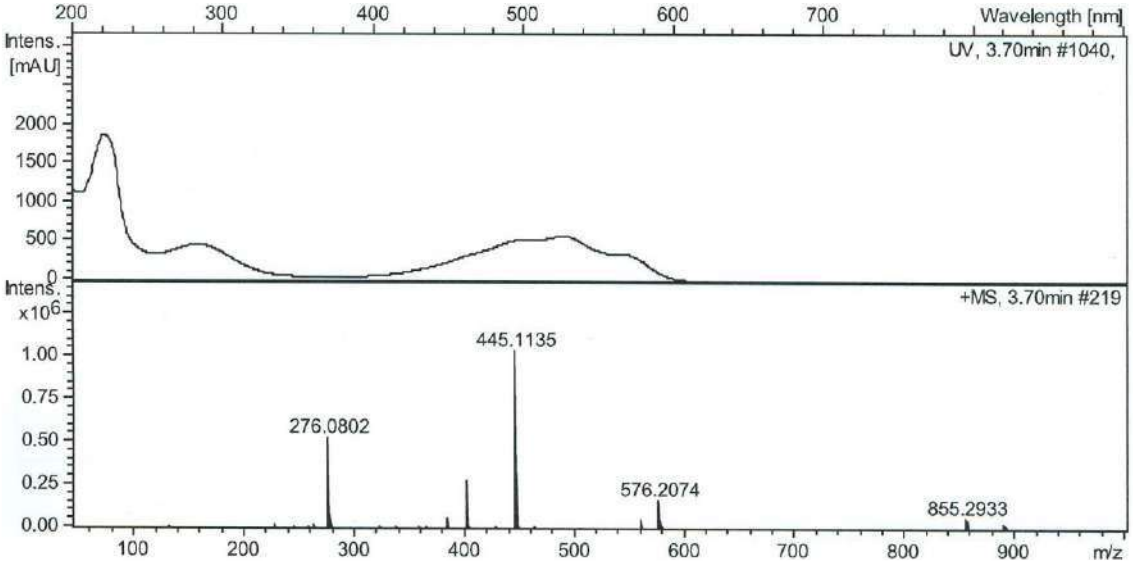


Granaticin A, $C_{22}H_{20}O_{10}$:



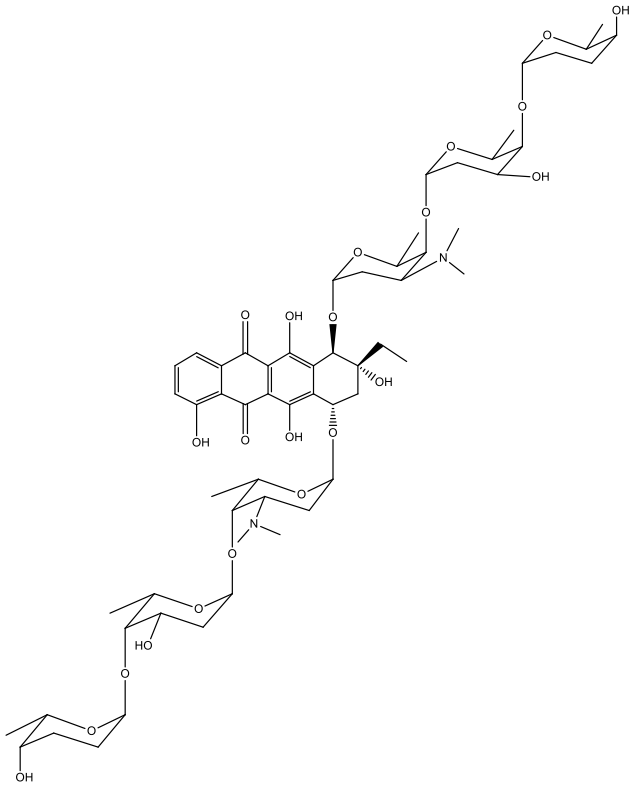
Granaticin C, $C_{28}H_{30}O_{12}$:

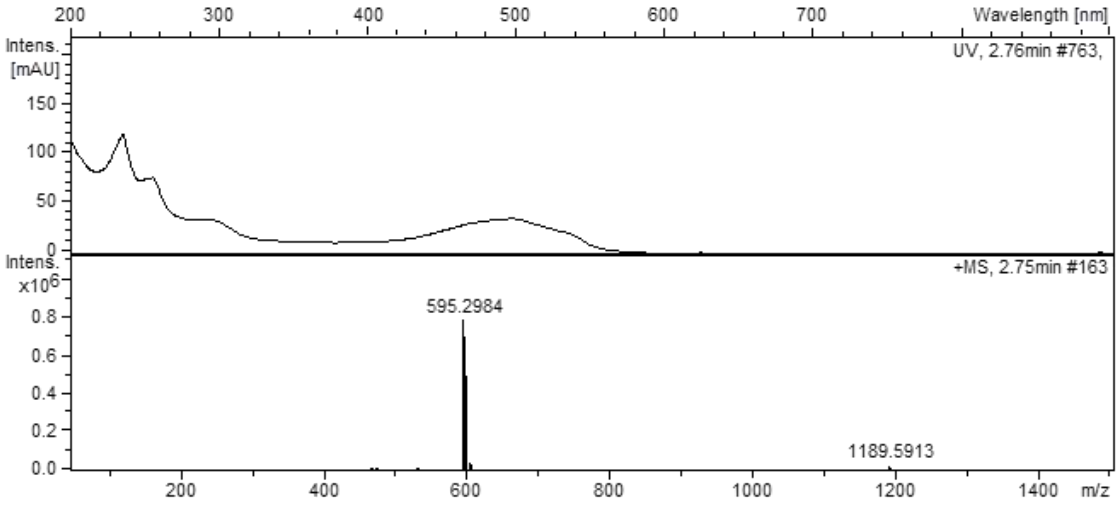




CS081a:

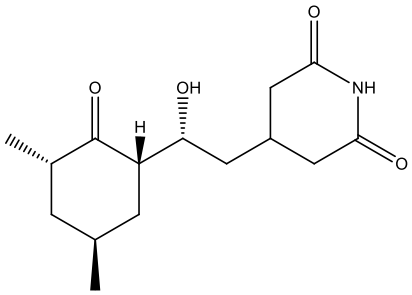
Cosmomycin D, $C_{60}H_{88}N_2O_{22}$:

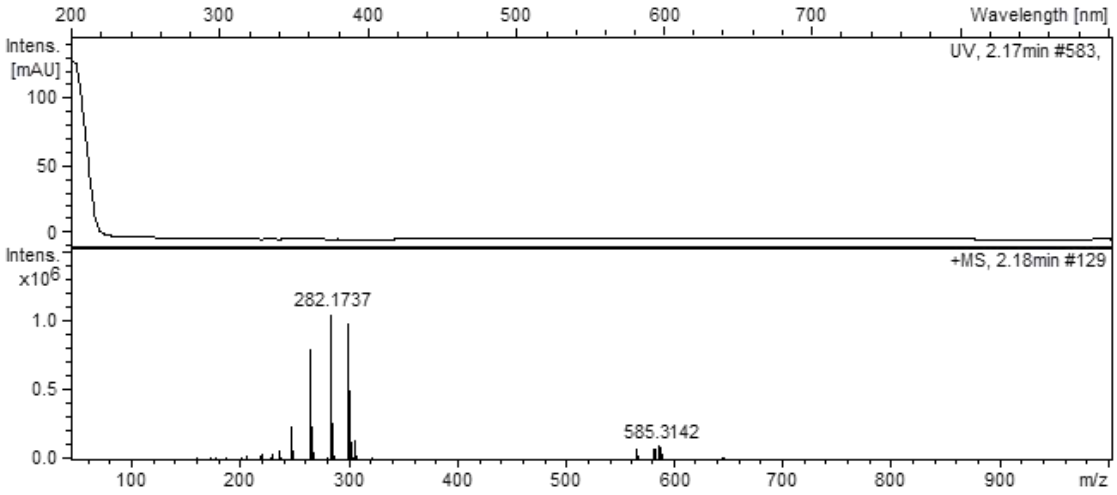




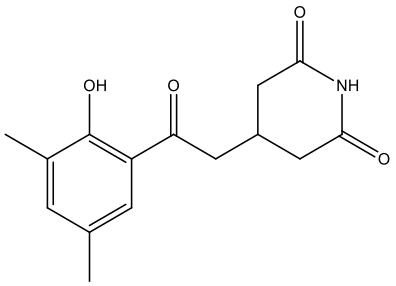
CS057:

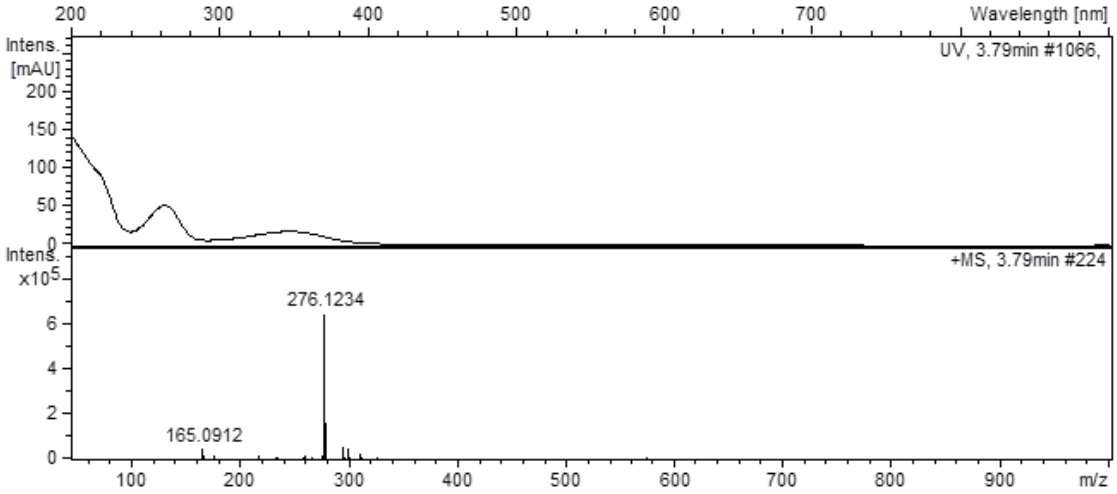
Cycloheximide, $C_{15}H_{23}NO_4$:





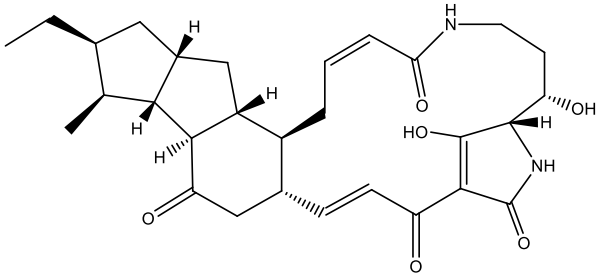
Actiphenol, $C_{15}H_{17}NO_4$:

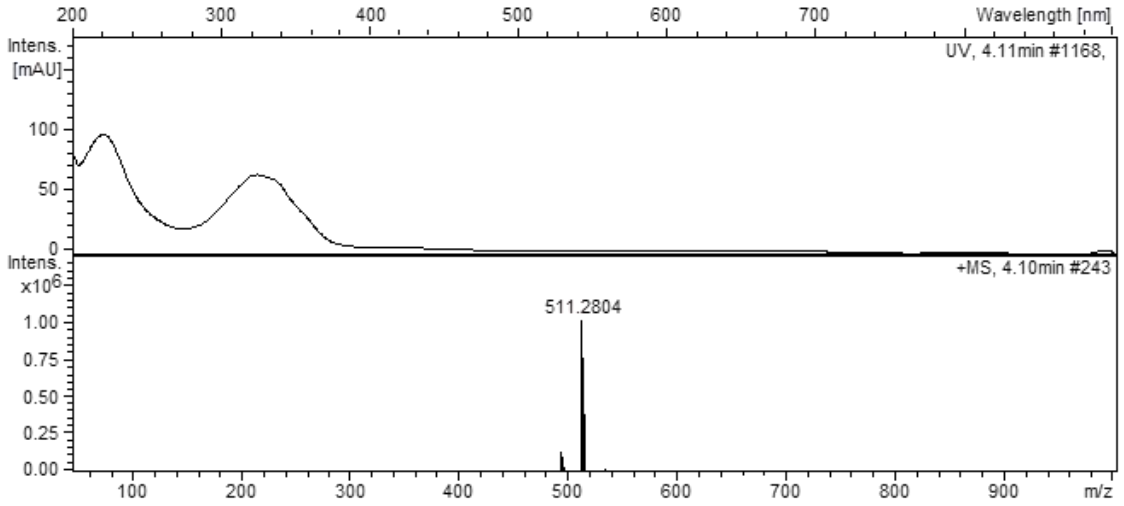




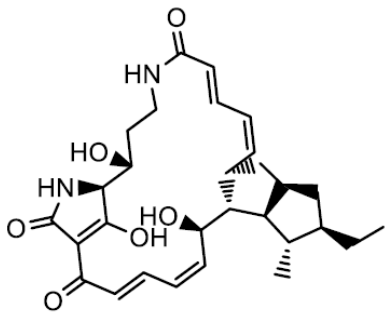
CS090a:

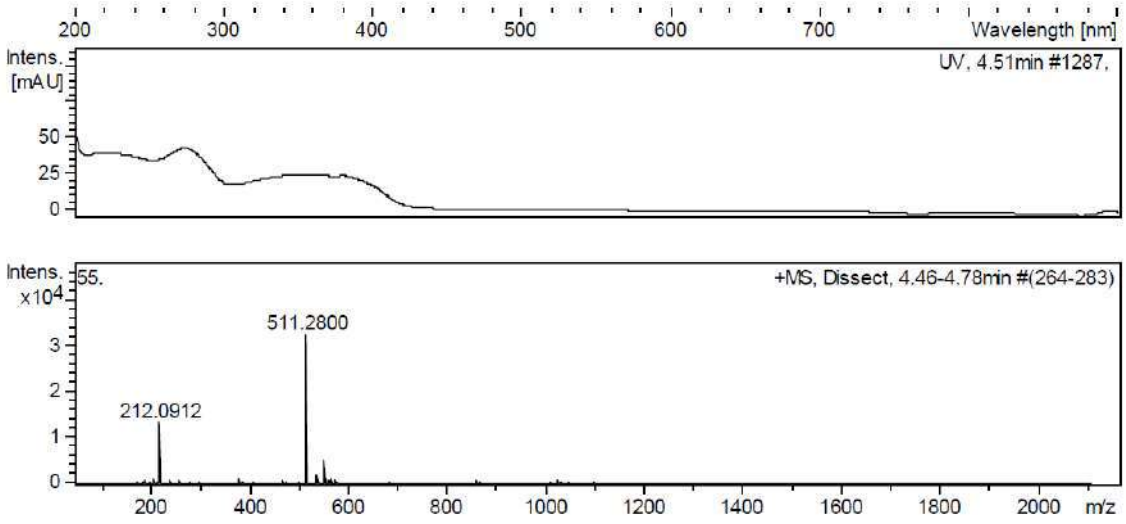
Maltophilin, $C_{29}H_{38}N_2O_6$:





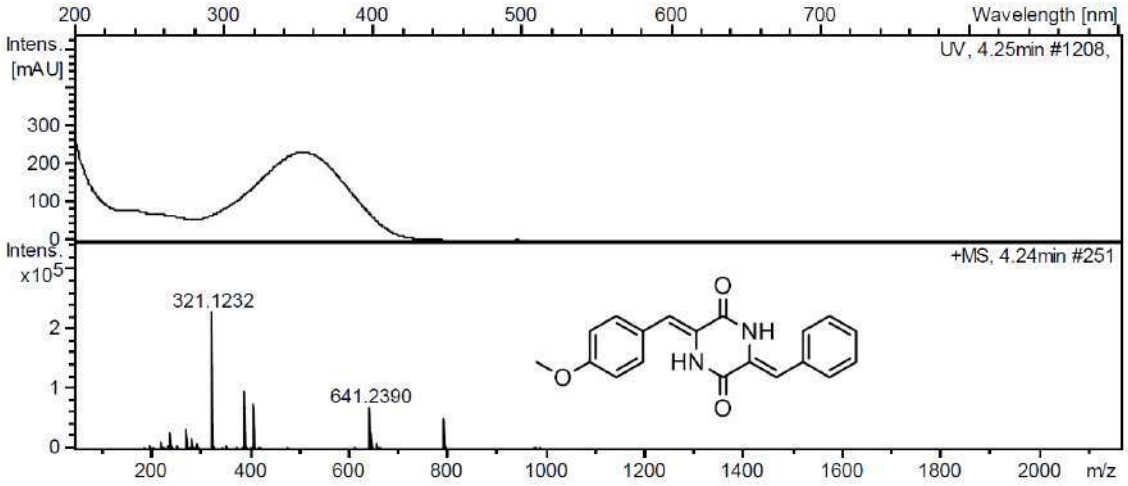
Alteramide A, $C_{29}H_{38}N_2O_6$:



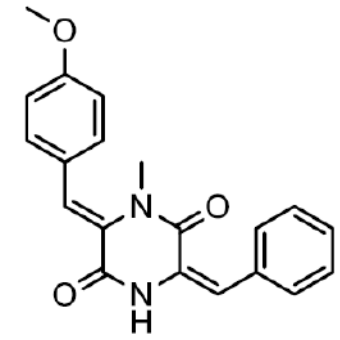


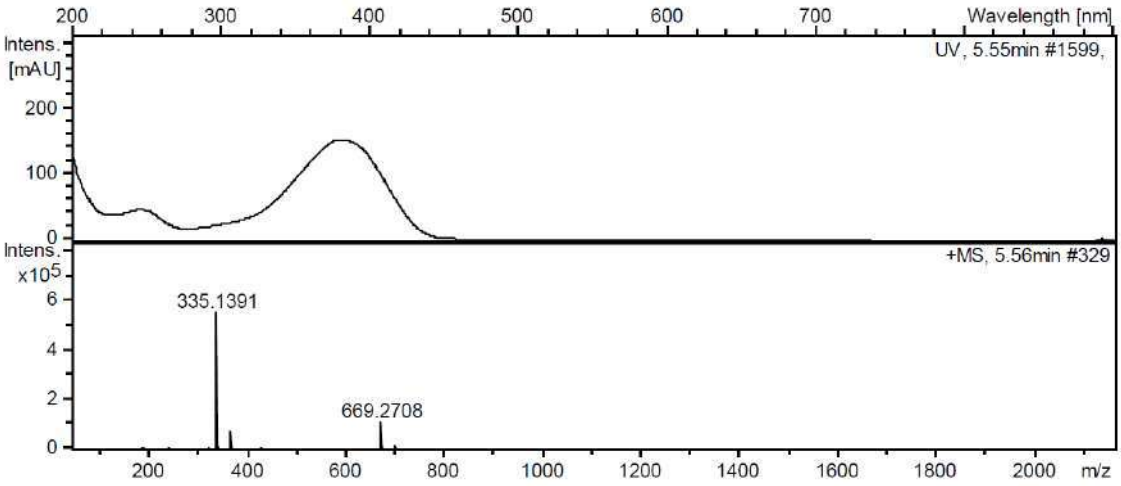
CS194:

Piperazinedione XR 334, $C_{19}H_{16}N_2O_3$:



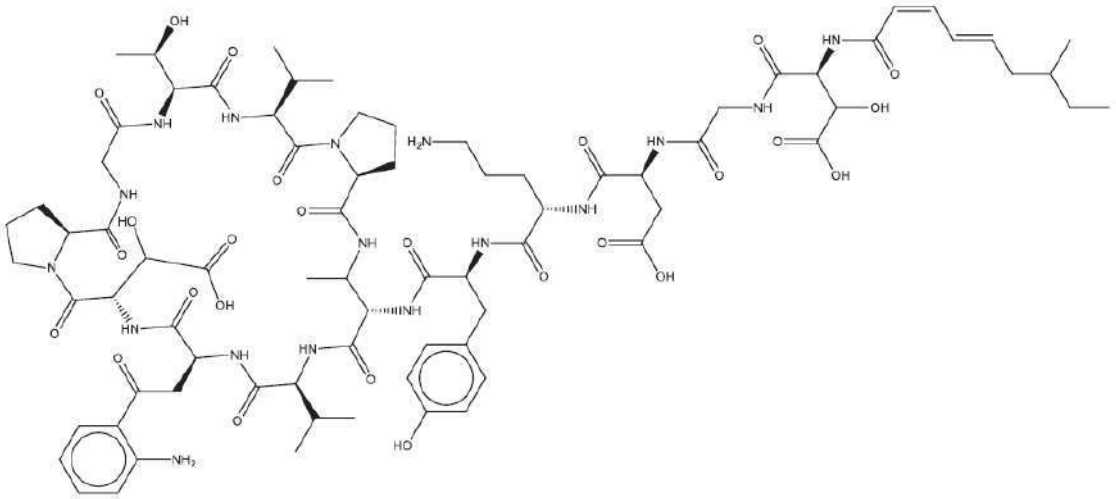
Piperazinedione XR 330, $C_{20}H_{18}N_2O_3$:

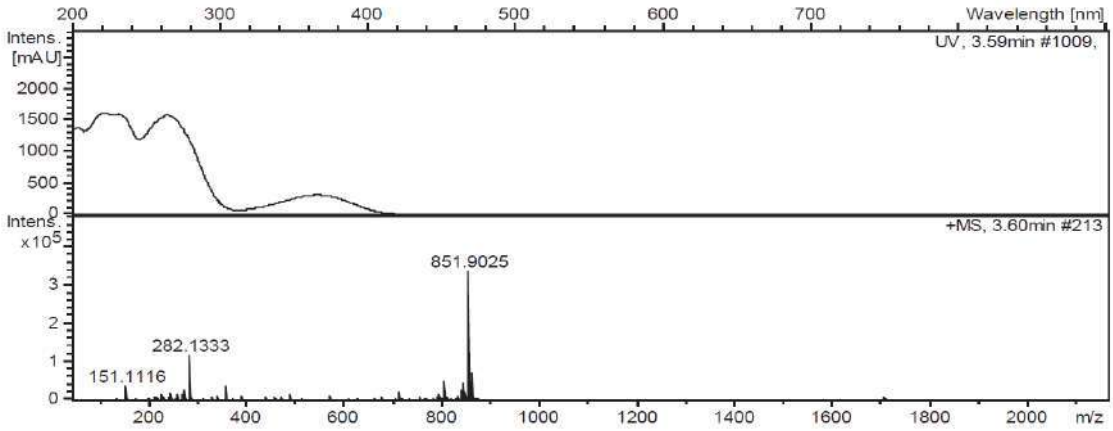




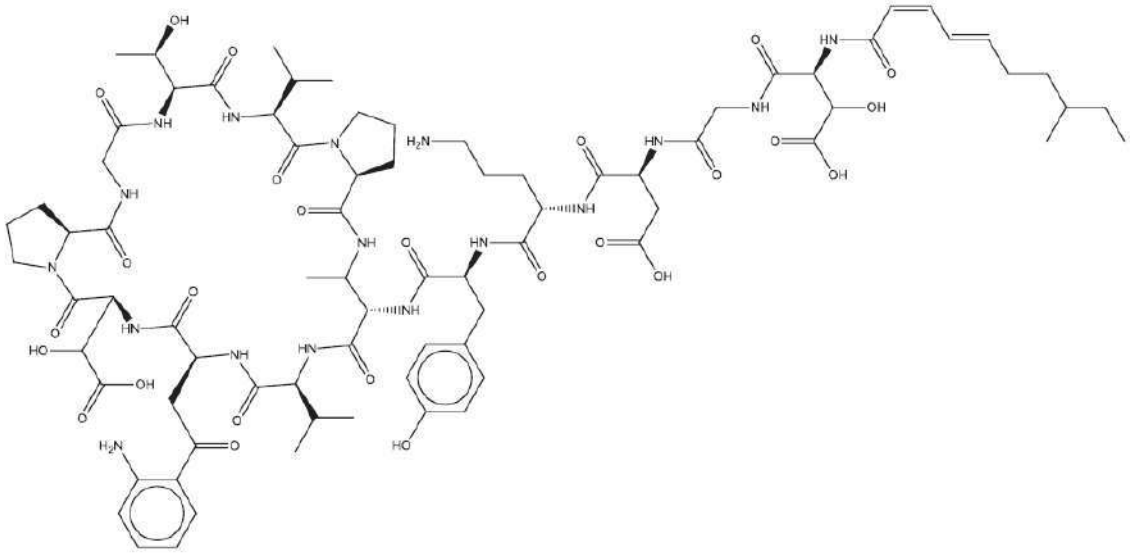
CS149:

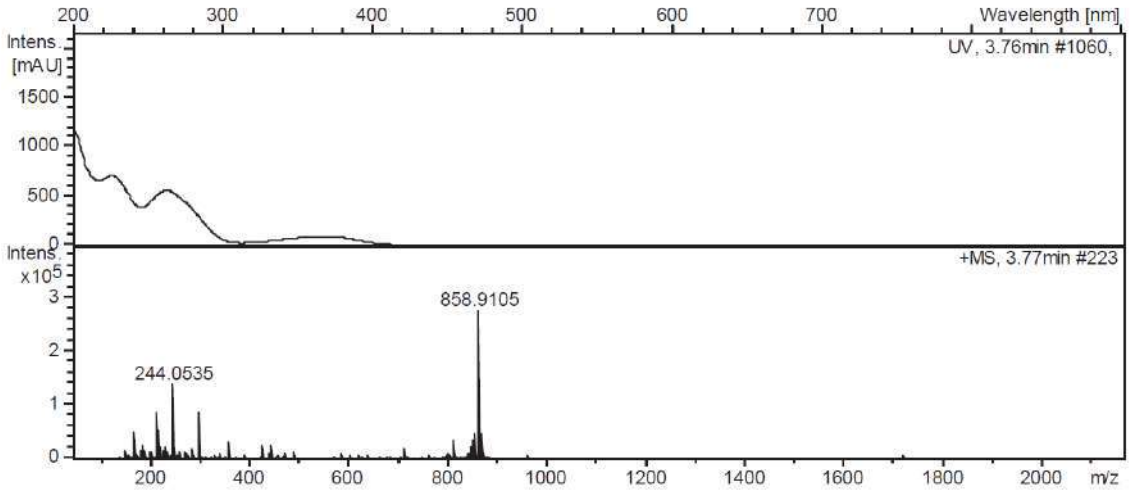
Rumycin 1, $C_{78}H_{111}N_{17}O_{26}$:





Rumycin 2, $C_{79}H_{113}N_{17}O_{26}$:

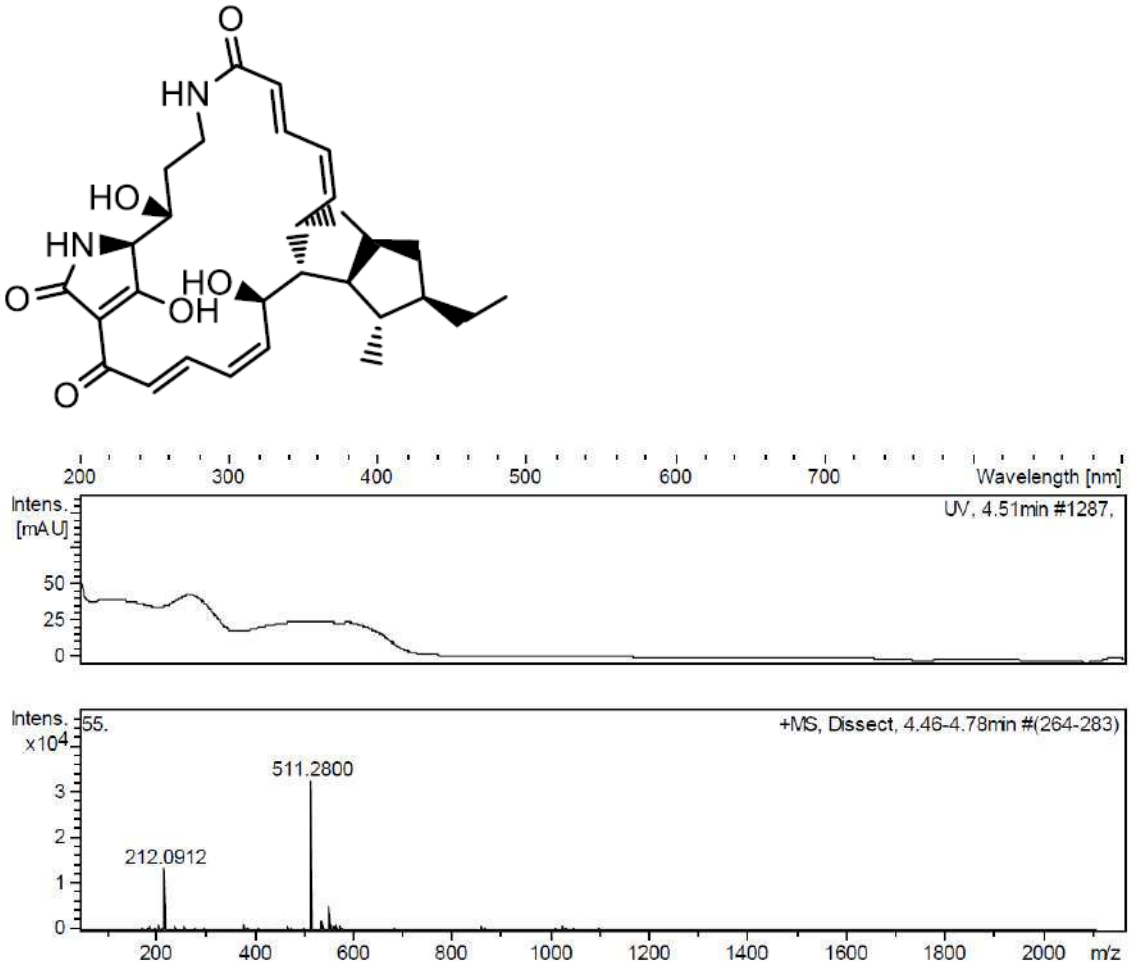




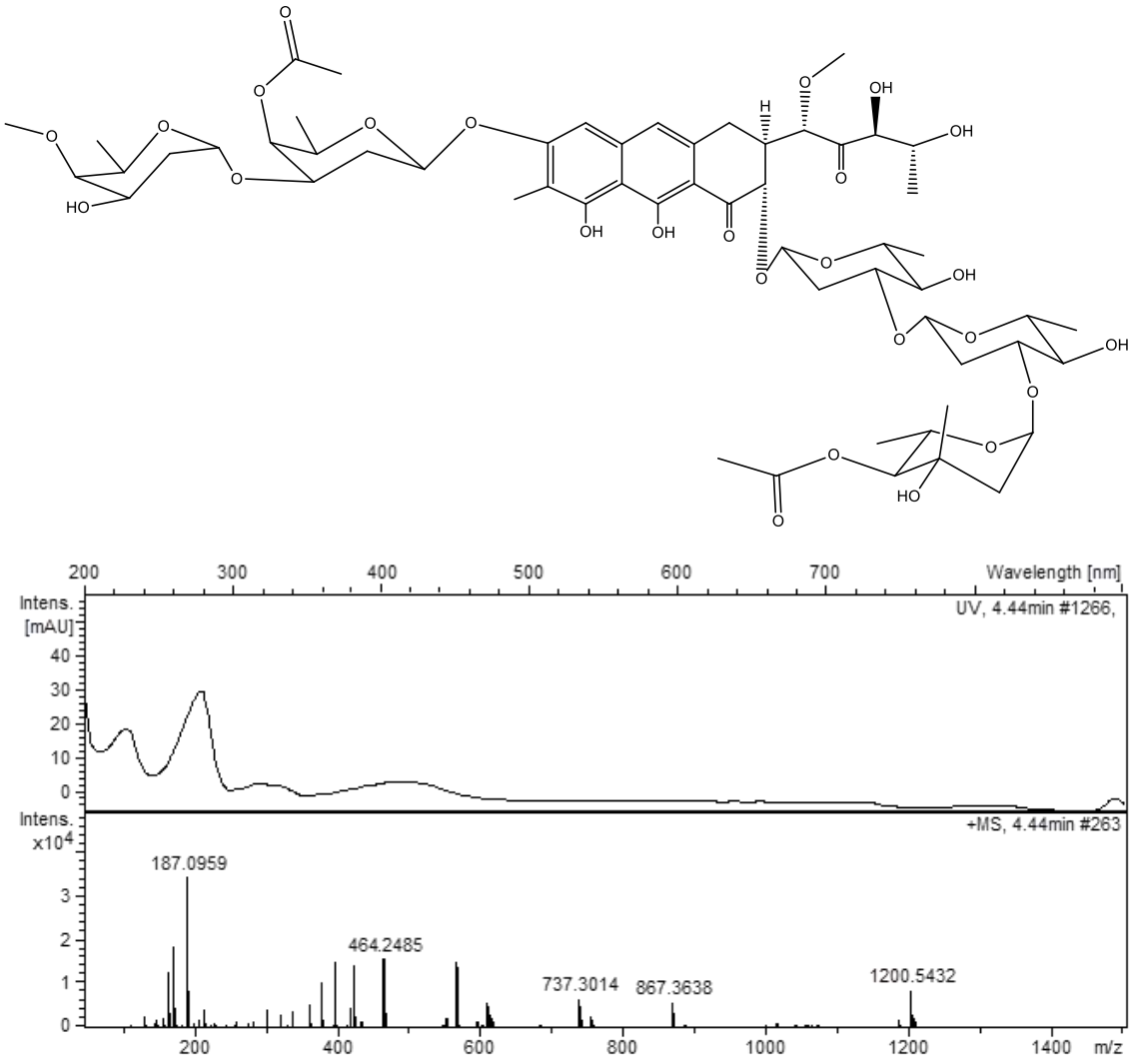
Section 3.3. Effect of VOCs in multiple co-culture

CS065a:

Alteramide A, $C_{29}H_{38}N_2O_6$:



Chromomycin A3, $C_{57}H_{82}O_{26}$:



Overview of the dereplication analysis.

Compound	**Molecular formula**	**Measured mass**	**Calculated mass**	**Most prominent ions**	**UV max (nm)**
Collismycin A-B	$C_{13}H_{13}N_3O_2S$	276.0821	275.33	276.0821 605.0723	249,5 332,7 469,8
Collismycin C	$C_{13}H_{14}N_2O_2S$	263.0850	262.33	263.0850	225,0 249,5 332,7 469,8

Collismycin D	$C_{13}H_{11}N_3OS$	258.0696	257.32	258.0696 574.1917	225,0 332,7 469,8
Granaticin A	$C_{22}H_{20}O_{10}$	445.1136	444.39	276.0801 445.1136 906.2452	215,3 280,9 494,2
Granaticin C	$C_{28}H_{30}O_{12}$	576.2074	558.54	276.0802 445.1135 576.2074 855.2933	215,3 280,9 494,2
Cosmomycin D	$C_{60}H_{88}N_2O_{22}$	1189.5913	1189.36	595.2984 1189.5913	235,4 255,0 295,0 493,5
Cycloheximide	$C_{15}H_{23}NO_4$	282.1737	281.35	282.1737 585.3142	200,0
Actiphenol	$C_{15}H_{17}NO_4$	276.1234	275.30	165.0912 276.1234	216,5 262, 4 345,2
Maltophilin	$C_{29}H_{38}N_2O_6$	511.2804	510.63	511.2804	222,6 322,8
Alteramide A	$C_{29}H_{38}N_2O_6$	511.2800	510.63	212.0912 511.2800	273,5 360,1
Piperazinedione XR 334	$C_{19}H_{16}N_2O_3$	321.1232	320.35	321.1232 641.2390	241,0 334,6
Piperazinedione XR 330	$C_{20}H_{18}N_2O_3$	335.1391	334.38	335.1391 669.2708	241,0 334,6
Rumycin 1	$C_{78}H_{111}N_{17}O_{26}$	851.9025	1702.84	151.1116 282.1333 851.9025	231,2 264,4 365,1
Rumycin 2	$C_{79}H_{113}N_{17}O_{26}$	858.9105	1716.87	244.0535 858.9105	226,9 261.8 365,7
Chromomycin A3	$C_{57}H_{82}O_{26}$	1200.5432	1183.26	187.0959 464.2485 737.3014 867.3638 1200.5432	229,3 277,8 317,3 403.7

Capítulo III

The Volatile Organic Compounds of *Streptomyces* spp.: An In-Depth Analysis of Their Antifungal Properties

microorganisms

MDPI

Article

The Volatile Organic Compounds of *Streptomyces* spp.: An In-Depth Analysis of Their Antifungal Properties

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Abstract: The study of volatile organic compounds (VOCs) has expanded because of the growing need to search for new bioactive compounds that could be used as therapeutic alternatives. These small molecules serve as signals to establish interactions with other nearby organisms in the environment. In this work, we evaluated the antifungal effect of VOCs produced by different *Streptomyces* spp. This study was performed using VOC chamber devices that allow for the free exchange of VOCs without physical contact between microorganisms or the diffusible compounds they produce. Antifungal activity was tested against *Escovopsis weberi,* a fungal pathogen that affects ant nest stability, and the results showed that *Streptomyces* spp. CS014, CS057, CS131, CS147, CS159, CS207, and CS227 inhibit or reduce the fungal growth with their emitted VOCs. A GS-MS analysis of volatiles produced and captured by activated charcoal suggested that these *Streptomyces* strains synthesize several antifungal VOCs, many of them produced because of the presence of *E. weberi,* with the accumulation of various VOCs determining the growth inhibition effect.

Keywords: *Streptomyces*; antifungal; GS-MS; *Escovopsis weberi*; volatile organic compounds; secondary metabolism

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1. Introduction

Actinomycetes are a group of Gram-positive bacteria commonly found in soils. Soil is a complex habitat exposed to highly variable conditions and colonized by a great number of species that interact and compete with each other. In this scenario, intra- and interspecific interactions play a key role in the stability and evolution of the biological community living below ground. The most studied interactions are based on the secretion of water-soluble compounds that can act as attractants, serve as alarm signals against predators, confer a competitive advantage, or even kill the recipient organisms [1] Among the Actinomycetes, bacteria of the genus *Streptomyces* stand out from a biotechnological point of view because of their enormous biosynthetic potential [2,3]; in fact, they are responsible for the production of many of the bioactive compounds used today in human and animal health [4]. *Streptomyces* has evolved over time to adapt and survive in such a competitive environment by producing a number of specialized metabolites. To overcome abiotic stresses, the vast majority of *Streptomyces* species produce siderophores, which provide them with a nutritional advantage over other organisms by solubilizing soil metals, thus increasing their availability, photoprotective compounds (such as melanin or carotenes), and osmoprotective metabolites (such as ectoin) [5]. Another competitive advantage of these bacteria is their significant ability to produce a wide array of diffusible compounds with antibacterial, antifungal,

and antiviral activity (e.g., cosmomycin, nystatin, and valinomycin, respectively). These metabolites, with different biosynthetic origins, allow them to compete for space and nutrients and colonize new environments by displacing previously established species [6].

Since most soils are not saturated with water, the dispersion of water-soluble compounds is restricted to short distances. Indeed, the large number of air-filled soil pores suggests the important role volatile organic compounds (VOCs) can play in this habitat, covering much greater distances than soluble compounds [7]. Volatiles comprise a wide range of metabolites belonging to different chemical classes but with common features: low molecular mass, high vapor pressure, a low boiling point, and a lipophilic nature [8]. In recent years, the study of microbial volatile organic compounds (MVOCs) has drawn the attention of the research community to their potential use in the biocontrol of agricultural pests, and the number of identified VOCs is rapidly increasing [9]. *Streptomyces* spp. are well-known producers of VOCs. Systematic surveys were conducted that led to the identification of more than 1400 VOCs produced by *Streptomyces* spp. belonging to different chemical classes and exerting diverse activities [6,10,11]. MVOCs are involved in important phenomena like quorum sensing/quenching, antibiosis, and/or communication; they can be assimilated into organic matter or even influence physiological processes (e.g., nitrification) [12,13].

The study of microbial volatiles is a promising new field that has suffered because of a lack of standardized, affordable, and simple equipment and methodologies because of the intrinsic nature of these compounds. Two main approaches are used to study MVOCs: passive diffusion systems, where volatiles diffuse freely into the headspace, and dynamic air stream systems, where compounds are funneled from the producer to the receiving organism, each with its own advantages and disadvantages [14]. Within the passive systems applied to bacterial VOCs, the divided Petri dish, plate-within-plate, and "sandwiched" Petri plate methods are the most used. Although they have been successfully applied in many studies, these methods have some drawbacks because of the high risk of cross-contamination and a lack of homogeneity and reproducibility in the results, and they can be difficult to set up. In this regard, new devices called volatile organic compound chambers (VOC chambers) were recently developed to solve these problems when working with microbial VOCs and demonstrated their usefulness and reliability in studying the antifungal VOCs produced by the filamentous fungus *Trichoderma* sp. [15]. This system was also successfully tested using *Streptomyces* spp. in our laboratory [16].

The urgent need for new bioactive compounds has led to the search for new producers in underexplored environments or symbiosis with other organisms [17,18]. Leafcutter ants are known to establish a complex relationship with various bacteria that help them fight the pathogenic fungus *Escovopsis weberi*. Bacteria of the genus *Pseudonocardia* have been described as true symbionts of these ants. However, a wide variety of *Streptomyces* strains have been found to co-inhabit ant nests. Much work has been performed describing the ability of these *Streptomyces* spp. to produce bioactive compounds (e.g., candicidins, actinomycins, sipanmycins) that could be added to those produced by *Pseudonocardia* to defend nests against other microorganisms [18,19]. The nests of these ants have a complex architecture made up of different underground chambers. This closed environment seems to be an ideal scenario for volatile compounds to have great relevance.

We hypothesize that VOCs emitted by *Streptomyces* sp. strains isolated from leafcutters impair the development of the pathogenic fungus *E. weberi.* We used the VOC chamber developed by Álvarez-García and co-workers to study VOCs produced by physically separated species and applied it, for the first time, to the analysis of interactions between *Streptomyces* spp. And *E. weberi*, mediated by volatile compounds. We found that several of these bacterial strains are capable of inhibiting (or delaying) the growth of the fungus in vitro. Also, we analyzed the VOCs present during that interaction using GC-MS and identified the possible volatile compounds responsible for the observed antifungal activity.

2. Materials and Methods

2.1. Strains and Culture Conditions

Streptomyces sp. strains used in this work belong to an in-lab CS collection (named after Carlos Sialer) isolated from the cuticle of leafcutter ants from the tribe *Attini* [19,20]. Strains were routinely grown on MA plates (Medium A) [21]) and incubated at 28 °C for 7 days. For metabolite production, strains were grown on either R5A [22] or soy flour mannitol medium (SFM) [23] agar plates. The pathogenic fungi *E. weberi* strain E and *Sclerotinia* spp. were cultivated at 28 °C on PDA plates (Potato Dextrose Agar, Oxoid), allowing for confluent growth, and then spread onto a fresh PDA plate with a cotton wool bud. For *Phytophthora cinnamomi* culturing, Sabouraud (Scharlab) medium was used. For multiple cocultures, SFM and YMA media (yeast extract, 3 g; malt extract, 3 g; peptone, 5 g, and glucose, 10 g per liter) were used.

2.2. Cocultured Streptomyces spp.–E. weberi

Spores of *Streptomyces* spp. were spread on half an R5A, SFM, or PDA plate. At the extreme of the other half of the plate, an agar plug of grown *E. weberi* was set as a fungi inoculum. After 5 days of incubating at 28 °C, we observed whether the fungus exceeded the confrontation line. Each experiment was performed in triplicate. The same experiment was repeated using *E. weberi* agar plugs confronted with two-day-grown *Streptomyces* spp.

2.3. Dual-Cultured Streptomyces spp.–E. weberi in VOC Chambers

Streptomyces sp. strains were grown on R5A or SFM plates at 28 °C for two days. An agar plug from the edge of a growing colony of *E. weberi* was placed in the center of a PDA plate, and immediately, a VOC chamber device was mounted as follows: a vented central piece was placed on top of the PDA plate (with the fungal agar plug facing up), and the plate with the two-day-grown *Streptomyces* spp. was placed upside down on top of it (Figure 1). The chamber device had a hole in the central part (without any type of film or filter covering it), allowing for the free exchange of VOCs between the cultures and avoiding physical contact between the strains or with the compounds that diffuse into the medium. The assembled VOC chamber was sealed with Parafilm® (Bemis, E-Thermo Fisher Scientific, Madrid, Spain). The dual culture was incubated at 28 °C for 5 days, and the diameter of the fungal colony was recorded after 2 and 5 days.

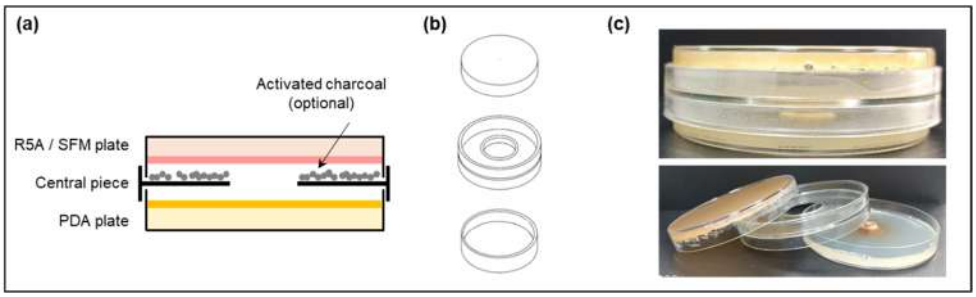

Figure 1. Volatile organic compound (VOC) chamber. (**a**) Schematic side view of a VOC chamber; (**b**) representation of each part of the VOC chamber device. The hole in the middle allows for the exchange of VOCs between cultures (modified from [16]); (**c**) photographs of an assembled VOC chamber (without charcoal).

The *E. weberi* colony diameter (in mm and measured in two different directions) was considered the mean value of the aforementioned two measurements. The percentage of fungal growth inhibition (GI) was calculated following the formula %GI = [(C − T)/C] × 100, where C is the colony size cultivated in the control plate, and T is the colony size measured in the VOC chamber. Each experiment was conducted in triplicate (n = 3). Control VOC chambers were also set without the *E. weberi* or the *Streptomyces* sp. strains.

2.4. Capture of VOCs and GC-MS Analysis

To analyze the compounds produced by the dual culture in SFM or R5A media, 250 mg of activated charcoal (Norit®) was placed on top of the chamber´s central piece, aiming to capture the emitted VOCs. This methodology was also applied to analyze VOCs produced by a single culture of *E. weberi* and a single culture of *Streptomyces* spp. in SFM or R5A media (only for strains CS057, CS131, CS014, and CS147). A VOC environmental control, an empty plate with active carbon, was also arranged. After 5 days of incubation at 28 °C, the activated charcoal was removed, and the captured volatiles were extracted with 750 µL of ethyl acetate. Also, an ethyl acetate sample was analyzed as a control to evaluate the volatile compounds that could be present in the solvent. Afterward, these samples were analyzed via gas chromatography–mass spectrometry (GC-MS) using an Agilent Technologies 7890A GC System coupled with a 5975C Inert XL MSD mass spectrometer in SCAN mode. Gas chromatography was carried out on an Agilent DB-5MS column (30 m × 0.25 mm × 0.25 µm with helium as carrier gas at 1 mL/min). The initial oven temperature was 50 °C, held for 5 min, ramped at 5 °C/min up to 300 °C, and held for 20 min. MassHunter Unknowns Analysis software and the NIST20 mass spectral library were used to identify compounds with a high percentage of reliability.

2.5. Data Analysis

Compounds identified with more than 70% reliability were selected. In order to create graphics, Rstudio and UpsetR package were used.

2.6. Dual Cultures against Other Fungi

An agar plug from the edge of a growing colony of *Phytophthora cinnamomic* or *Sclerotinia* spp. was placed in the center of a Sabouraud agar and PDA plates, respectively. The VOC chamber device was mounted as previously described. Two-day-grown *Streptomyces* spp. were placed upside down on top of the chamber.

2.7. Analysis of Diffusible Compound Production

In total, 3.5 g of *Streptomyces* sp. agar plates grown on SFM medium cocultures with *E. weberi* or *P. cinnamomi* were extracted with 5 mL of different organic solvents (ethyl acetate, ethyl acetate containing formic acid (1%), or butanol) and analyzed via reverse phase chromatography in a Waters Acquity UPLC instrument fitted with a BEHC18 column (1.7 µm, 2.1 mm × 100 mm; Waters, Milford, MA, USA), with acetonitrile and MQ water + 0.1% trifluoroacetic acid (TFA) as the mobile phase. The photodiode array (PDA) detector was set to scan wavelengths between 200 and 600 nm. The samples were eluted with acetonitrile (10%) for 1 min, followed by a linear gradient of acetonitrile (10–100%) for 7 min (flow rate of 0.5 mL/min; column temperature, 35 °C). As a control for the assay, *Streptomyces* sp. agar plates without confrontations from any microorganisms were extracted.

2.8. Multiple-Cultured Streptomyces spp.–E.weberi

Two different *Streptomyces* spp. were cultured on small YMA or SFM Petri dishes (5 cm diameter). After 48 h, both plates and a small PDA plate containing an agar plug from the edge of a growing colony of *E. weberi* were placed open inside a large Petri dish (diameter of 13.5 cm). This device was sealed with Parafilm® (Bemis, E-Thermo Fisher Scientific, Madrid, Spain) and incubated at 28 °C for 5 days (Figure S1, Supplementary Material). The diameter of the fungal colony was recorded after 2 and 5 days. As a control, one *Streptomyces* sp. plate and an *E. weberi* plate were settled on a large one for confrontation. Moreover, an *E. weberi* dish alone was cultured on a large plate. Each experiment was conducted in triplicate.

2.9. pH Variation Analysis

To test the variation in the pH of the device environment as a consequence of the effect caused by VOCs, three different small and opened plates were placed inside a large one: an SFM *Streptomyces* sp. plate, a PDA plate with a plug of *E. weberi,* and a PDA + phenol red broth (Condalab) plate as a pH indicator. This device was incubated at 28 °C for 5 days. As a control, a *Streptomyces* sp. plate was cultured at 28 °C for 5 days in the presence of one plate containing the indicator. In addition, a single indicator plate and an *E. weberi* plate were placed inside the large dishes and incubated at 28 °C for 5 days.

3. Results and Discussion

The broad relationship described between the *Attini* ant tribe and *Streptomyces* spp. has left evidence of the important role that *Streptomyces* spp. might play in their nests. Their great potential to produce bioactive compounds suggests a benefit that the entire community can obtain for the maintenance of the system's balance. One of the elements of the imbalance that threatens this system is the pathogen *E. weberi,* so it is worth investigating the involvement of *Streptomyces* in the biosynthesis of antifungal compounds that hold *E. weberi* at bay.

3.1. Cocultured Streptomyces spp.–E. weberi

This assay was performed in order to evaluate the effect of the coculture between *E. weberi* and different *Streptomyces* sp. strains of the CS collection. It was performed by confronting the fungus and the bacteria at the same time and confronting *E. weberi* with a two-day-grown *Streptomyces* sp. to evaluate if there are differences between the two setups.

The results of this experiment were variable. Three media were used: R5A and SFM, rich media for the growth of *Streptomyces* spp., and PDA, a suitable medium for *E. weberi* growth. However, a diversity of results was perceived depending on the strain, media, and whether the *E. weberi* plug was inoculated at the same time as the *Streptomyces* spp. or in a two-day-grown plate. This difference is based on the stationary phase of the two-day-grown *Streptomyces* spp., which allows these bacteria to produce secondary metabolites with possible antifungal properties [24]. However, in cases where both microorganisms are inoculated at the same time, this advantage of the *Streptomyces* sp. strain does not exist, and therefore, *E. weberi*, which grows faster, tends to overgrow and spread throughout the entire plate in most cases, even on top of the *Streptomyces* spp. culture (Figure S2, Supplementary Material).

In the case of cultures performed on PDA medium (which is more favorable for fungal growth), *E. weberi* grows throughout the entire plate, lysing most of the different *Streptomyces* sp. colonies tested, regardless of whether *Streptomyces* spp. were growing for 2 days before or not. In the case of two-day-grown CS159, this strain prevents *E. weberi* growth beyond the confronting line (the line delimiting the *Streptomyces* sp. growth zone). This fact does not occur when both microorganisms are grown at the same time, since *E. weberi* spreads over the entire surface of the plate. In the case of CS081a, CS147, and CS149 two-day-grown plates, a partial contention on the *E. weberi* growth can also be observed (Figure S2, Supplementary Material).

When the coculture occurs in R5A medium, it is favorable for *Streptomyces* spp. growth and the production of secondary metabolites because of the richness of its composition; the inhibition of *E. weberi* growth can be observed to a greater extent since it does not reach the confrontation line in any case (both in the plates with two-day-grown *Streptomyces* spp. and the ones with both microorganisms cultured at the same time). In some cases, it can be seen that this inhibition of growth is greater in the two-day-grown plates, for example, with strains CS090a and CS113 (Figure S2, Supplementary Material). This effect may be mainly due to the effect of diffusible compounds, as well as the volatiles produced by the *Streptomyces* sp. strains since they all have a great metabolic potential for the synthesis of antimicrobial compounds [5,25].

SFM is the medium that offers the most variable results. On the one hand, in cocultures with strains CS090a, CS113, CS207, and CS227, *E. weberi* exceeds the confrontation line, contrary to the results on R5A medium, where the growth of *E. weberi* is controlled by *Streptomyces* spp. This may be because the fungal growth on R5A may be more retarded or because antifungal metabolites that exert an inhibitory effect are biosynthesized in R5A and are not produced on SFM. However, in the majority of cases, the growth of *E. weberi* does not reach the confrontation line, but this inhibition is more pronounced in the case of the two-day-grown *Streptomyces* spp. (except for strains CS057, CS81a, and CS149). This seems to be logical; since the production of potential antifungal compounds by *Streptomyces* spp. occurs before the incorporation of *E. weberi* and therefore the inhibition is observed to a greater extent. In the case of the aforementioned exceptions, this effect is probably due to the production of variable compounds in the different stages of *Streptomyces* development (Figure S2, Supplementary Material) [26].

Based on these results, interest in the implications of volatile compounds in this assay increases. Indeed, in the natural environment in which these microorganisms coexist, both liquid and volatile compounds can be responsible for exerting an effect on the pathogen. However, it might also be true that the two types of microorganisms are not necessarily always in physical contact with each other, or the diffusible compounds do not have enough range of activity to affect *E. weberi,* so the effect of volatile compounds may play a more important role that we initially imagined. For that reason, a study of the effect of the volatile compounds of *Streptomyces* spp. on *E. weberi* was planned, with no contact between either microorganism or with the soluble compounds that they disseminate.

3.2. Dual-Cultured Streptomyces spp.–E. weberi in VOC Chambers

Coculture trials of the pathogenic fungus *E. weberi* and various strains of *Streptomyces* spp. from the CS collection were performed with the aim of evaluating the potential of the ecological relationships that these species, coexisting in the same environment, can maintain.

The use of VOC chambers is useful in evaluating signaling and effects between these two microorganisms because of the exclusive effect of the VOCs since there is no physical contact between them or with the compounds that both can diffuse into culture media. This strategy is important in determining that, although both species live in the same environment, there may not be physical contact between them, but the signaling relationships between them are maintained through volatiles, which can trigger different types of effects.

Specifically, in this test, we observed how certain *Streptomyces* strains growing on SFM medium inhibited or delayed the growth of the pathogen in comparison with the control sample, which was a plate of *E. weberi* in a VOC chamber without *Streptomyces* spp. Thus, *Streptomyces* spp. CS227, CS014, CS207, CS147, and CS159 delayed *E. weberi*'s growth at different levels, whereas CS131 and CS057 nearly inhibited its development (Figure 2). On the other hand, none of these strains produced antifungal compounds in a suitable quantity to make the effect visible when cultured on R5A. *E. weberi* is a fungus with very large aerial growth, which quickly invades all the available space after a few days of cultivation.

Based on these results, the inhibition percentage (Figure 3) was calculated by measuring the diameter of the colony. It can be seen that strains CS057, CS131, and CS227 cause growth inhibition in *E. weberi* above 90%.

This experiment is the first time that these chambers have been used for bacteria–fungus interaction tests since they were first employed for fungus–fungus [15], fungus–insect [27], fungus–plant [28], and bacteria–bacteria assays [16].

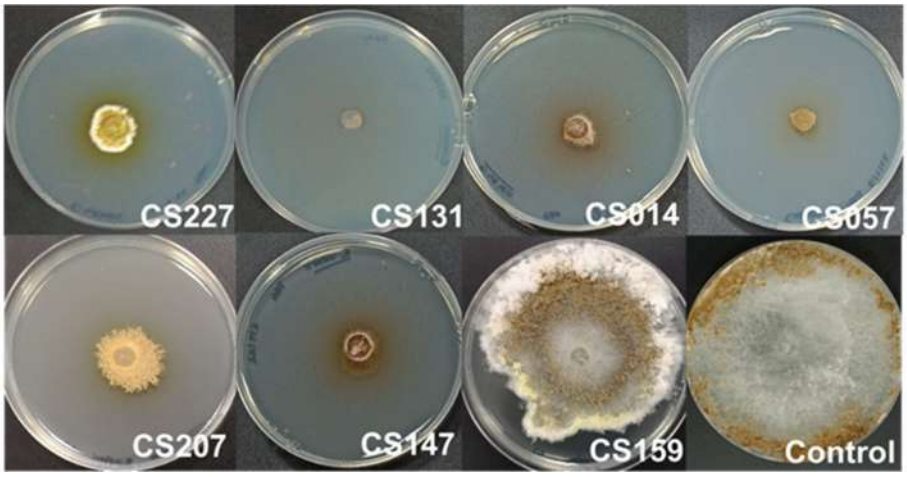

Figure 2. Antifungal effect of *Streptomyces*'s volatile organic compounds (VOCs) on *Escovopsis weberi*. From left to right, *E. weberi* plate when cocultured in VOC chambers against *Streptomyces* spp. CS227, CS131, CS014, CS057 (first row), CS207, CS147, and CS159 (second row) grown on soy flour mannitol (SFM) medium. As a control for the assay, the last image corresponds to a VOC chamber without a *Streptomyces* sp. culture.

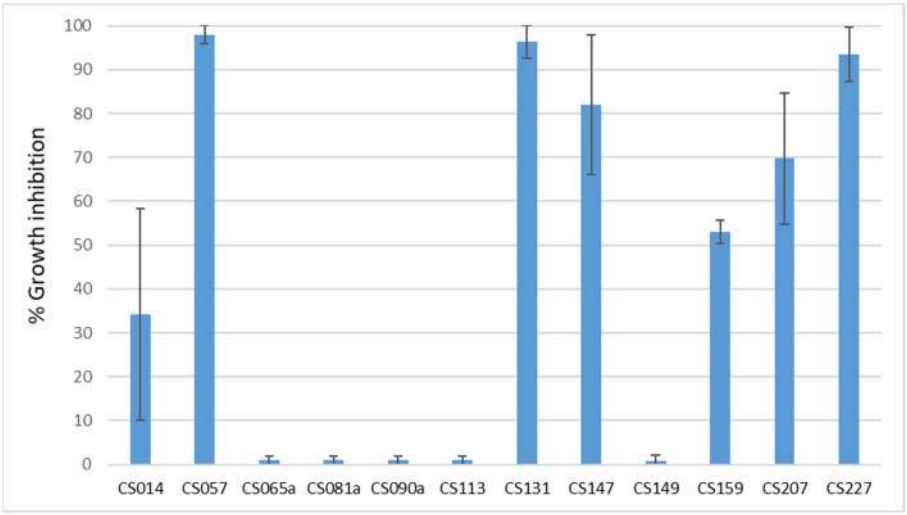

Figure 3. Average growth inhibition of *Escovopsis weberi* (n = 3) when cocultured with different *Streptomyces* sp. strains. Strains CS057, CS131, and CS227 cause inhibition above 90%. The standard deviation of the results is represented.

3.3. Capture of VOCs and GC-MS Analysis

The capture of volatiles emitted by the coculture was subsequently performed using activated charcoal. The compounds retained in activated charcoal were extracted with ethyl acetate and analyzed via GS-MS. After we positively identified the detected VOCs by comparing their MS spectra with those deposited in the NIST20 library, a clustering study was performed using the R software. In this assay, the Upset Plot is a data visualization method that shows, on a vertical axis, the number of compounds that are exclusively in each sample, the ones that are present in the various samples, and indicating in which. On the horizontal axis, the number of compounds identified in each sample is represented.

This assay was performed with four *Streptomyces* sp. strains (CS057, CS147, CS014, and CS131) on SFM and R5A media. Since the production of antifungal compounds does not occur when the strains are cultivated on R5A (according to Results Section 3.2), all compounds detected in common with the samples that are cultured in SFM are considered not responsible for the antifungal activity.

3.3.1. Cocultured *E. weberi–Streptomyces* sp. CS057

A total of 53 VOCs from those captured by activated charcoal and extracted with ethyl acetate were positively identified using the NIST20 library (Table S1, Supplementary Material), of which 40 of them were detected in the samples with *Streptomyces* sp. CS057 or its coculture with *E. weberi*. The Upset Plot (Figure 4) showed a wide variety of compounds known to be part of plant essential oils, most of them with bioactivities described in [29–31]. Nevertheless, most of these properties are described in essential oils, not in the isolated compounds that comprise them. For instance, calamenene is a component of several plant essential oils with antimicrobial, antiviral, anti-biofilm, and antioxidant activities [32–35]. S-methyl methanethiosulfonate and allyl nonanoate are related to significant antifungal and antibiotic activity, respectively [36,37]. Sabinene (Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-) is also part of plant oils and has a wide number of applications as flavorings, perfume additives, and biofuels [38]. Similarly, pyrazines have different antimicrobial properties in various applications in the food industry, agriculture, and pharmacy [39–41]. Special mention should be made of cubenene o 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)γ-cadinene((1S-cis)-Naphthalene, which has strong antitumoral activity against ovarian cancer [42] and antimicrobial bioactivity against different clinical pathogens [32,43], and this compound is identified in various samples in both media and in other strains. The other compounds identified were 2-methylisoborneol or trans-1,10-Dimethyl-trans-9-decalinol (commonly known as geosmin), typically produced by *Streptomyces* [44]. Among these VOCs, only three were exclusively produced and detected in confrontation samples on SFM (Table S2, Supplementary Material).

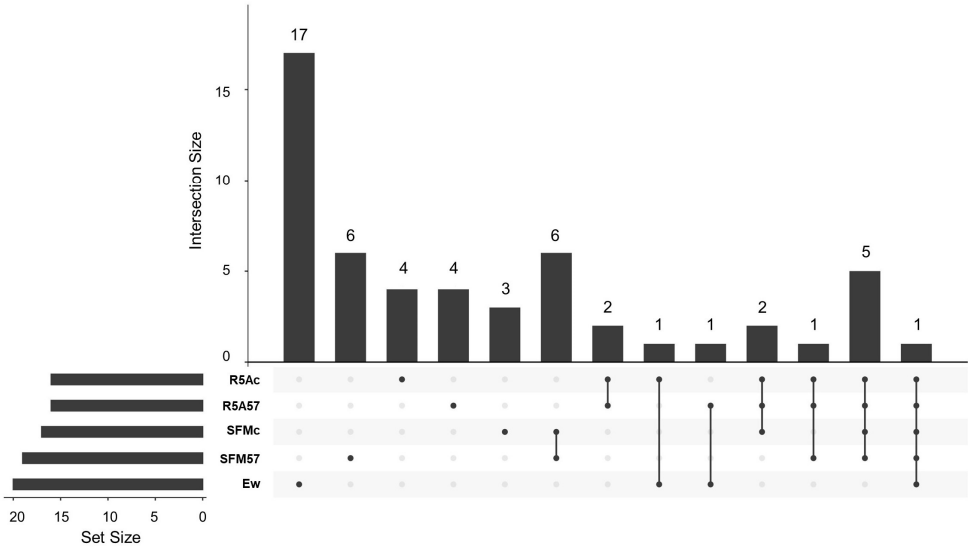

Figure 4. Upset Plot from the compounds detected via GS-MS from the assay confronting *Streptomyces* sp. CS057 and *Escovopsis weberi*. Ew: volatiles produced by *E. weberi*; SFMc: volatile produced during the coculture of *E. weberi–Streptomyces* sp. CS057 on soy flour mannitol (SFM) medium; SFM057: volatiles produced by CS57 on SFM; R5A57: volatiles produced by CS057 on R5A medium; R5Ac: volatiles produced during the coculture of *E. weberi–Streptomyces* sp. CS057 on R5A.

- **Camphene**: (2,2-dimethyl-3-methylidenebicyclo [2.2.1] heptane). This is a bicyclic terpene produced by various medicinal plants and associated with antibacterial, antifungal, antioxidant, anticancer, antiparasitic, antiviral, anti-inflammatory, and hypolipidemic activities [45]. The remarkable antifungal activity of this compound and its derivatives against diverse pathogenic fungi (*Candida albicans*, *Aspergillus flavus*, *Microsporum canis*, among others) [26,46] supports the hypothesis regarding the activity of this compound, which was exclusively present in the confrontation where antifungal activity was spotted.

- **4-methyl-1-(1-methylethyl)-Bicyclo[3.1.0]hex-2-ene**: (beta-thujene). This terpene has not been found to be produced by bacteria. It has been described as a plant metabolite associated with antifungal and antibacterial activity [47,48]. Despite the high percentage of reliability with which this compound has been identified (>90% in each replicate), we cannot assure the identification of this compound since no bibliographical information supports the bacterial production of beta-thujene. It is remarkable that other similar compounds (4-methylene-1-(1-methylethyl)-bicyclo[3.1.0]hexane and 6-hydroxy-5-methyl-6-vinyl-bicyclo[3.2.0]heptan-2-one) were detected in other samples, so we suggest that perhaps they are various modifications of the same compound caused by interactions with other volatiles that may contain this antifungal activity.
- **2-propenyl ester-octanoic acid**: Acids of this type are lipid residues from alcoholic fermentation that can be subsequently used as a substrate to produce other compounds. Oxalic acid has been reported to exert antifungal activity at certain levels by membrane disruption [49,50]. Although other fatty acids are present in these samples, octanoic acid is only present in SFM confrontation samples in a large enough quantity to be detected using the method followed, so it can be a degradation product that contributes to the VOC chamber environment conditions that affect fungal growth.

Accordingly, it is clear that the presence of *E. weberi* generates a response in *Streptomyces* metabolism. By studying the compounds that were detected via SFM confrontation and SFM-CS057 samples, we identified six compounds shared by both groups (Table S2, Supplementary Material).

2,6,6-Trimethyl (beta-homocyclocitral)1-cyclohexene-1-acetaldehyde [51] and 1,3,3-trimethyl-tricyclo [2.2.1.0(2,6)] heptane (cyclofenche) [52] are compounds with antifungal activities present in essential oils. Similarly, 4,8-dimethylnona-1,3,7-triene is also described as a monoterpene part of plant essential oils with antifungal activities [53]. 3-Octanone has been described as an antifungal cetone [54]. No bioactivity has been described for 3,6-heptanedione and trans 9-methylene-3-oxabicyclo [5.3.0] decan-2-one. These six molecules are common to both groups and contribute to the ones exclusively produced by CS057 on SFM, generating a hostile environment for the growth of *E. weberi*. In summary, the presence of this fungus generates a response in the metabolism of *Streptomyces*, leading to the production of octanoic acid and other VOCs.

In addition, taking into account the biosynthetic gene clusters present in the genome of *Streptomyces* sp. CS057 [5] (predicted using AntiSMASH [55]), of the potential production of terpene-type compounds, there are only two possible pathways responsible for the synthesis of the terpenes detected during this work. On one hand, cluster 1.31 might be responsible for the synthesis of geosmin since it has 100% similarity with the described cluster for the synthesis of this compound. This agrees with results observed at the experimental level since every sample extracted from CS057 cultures (in SFM, R5A, and confrontations against *E. weberi*) showed the presence of related compounds such as trans-1,10-dimethyl-trans-9-decalinol and other molecules with a naphthalene structure. In addition, cluster 1.10 is predicted to be responsible for the synthesis of terpene and has a 69% similarity with the corresponding cluster for hopene biosynthesis. Similarly, cluster 1.11 is predicted to be responsible for 2-methylisoborneol (100% similarity with the described cluster). On the other hand, cluster 1.15 (no similarity with the other described clusters) and cluster 1.26 (19% similarity with the steffimycin D cluster) could be responsible for the biosynthesis of some of the terpenes identified via GS-MS in this strain since terpene synthase/cyclase genes were identified on them (*ctg1_2020* and *ctg1_6135* respectively). Terpenoids are diverse natural compounds obtained from tailoring steps after initial products from terpene synthases, which explains the large number of terpenes identified in comparison with the number of clusters involved in their synthesis [56].

3.3.2. Cocultured *E. weberi*–*Streptomyces* sp. CS131

The assays confronting strain CS131 with *E. weberi* showed that there were sixty-one compounds captured by the activated carbon and extracted with ethyl acetate, detected

via GS-MS and identified using the NIST20 library (Table S3, Supplementary Material). Of these compounds, only seven were exclusively produced and detected in confrontation samples on SFM medium (Table S4, Supplementary Material). The other fifty-four were also produced by other samples (Figure 5). A broad number of properties have been described in the compounds identified. As expected, 2-methylisoborneol and geosmin were detected. Likewise, different compounds with antimicrobial activity were detected, such as furanones, dimethyl disulfide, 2,6,6-trimethyl-1-Cyclohexene-1-acetaldehyde, and muurola derivatives [53,57,58]. It is worth highlighting the production of caryophyllenyl alcohol, identified in several samples in both media and associated with several applications in cosmetics and derivatives in the drug industry (hypolipidemic, anti-inflammatory properties [59–62].

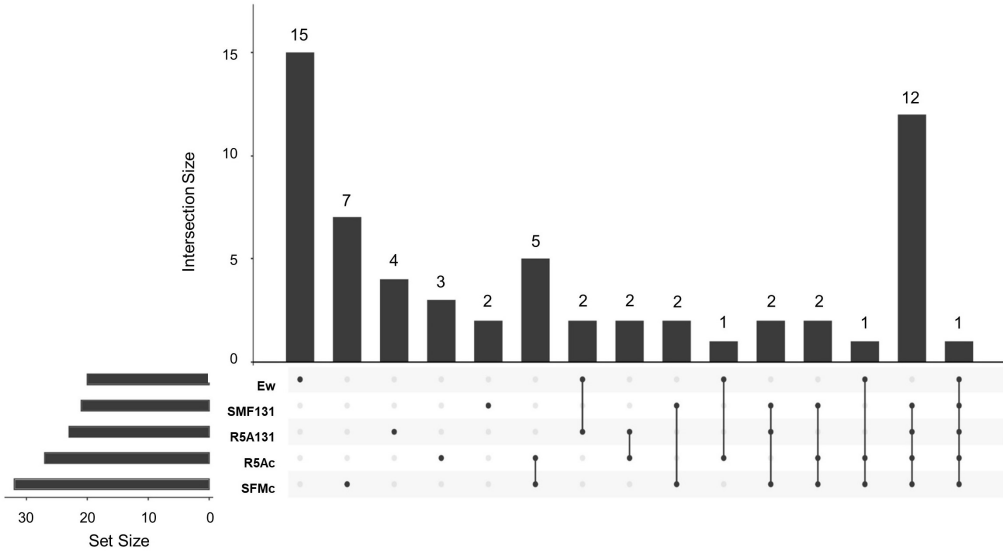

Figure 5. Upset Plot from the compounds detected via GS-MS from the assay confronting *Streptomyces* sp. CS131 and *Escovopsis weberi*. Ew: volatiles produced by *E. weberi*; SFMc: volatile produced during the coculture of *E. weberi–Streptomyces* sp. CS131 on soy flour mannitol (SFM) medium; SFM131: volatiles produced by CS131 on SFM; R5A131: volatiles produced by CS131 on R5A medium; R5Ac: volatiles produced during the coculture of *E.weberi–Streptomyces* sp. CS131 on R5A.

Those compounds that were exclusively produced via SFM medium confrontation demanded attention: no activity has been described for 1,2,4,5-Tetrazin-3-amine or bis(1,1,3,3-tetramethylbutyl) disulfide in the literature. However, antifungal activity was attributed to 1,2,4,5 tetrazines derivatives [63], some sulfides, and bis(1,1,3,3-tetramethylbutyl) derivatives [58]. 3-octanone and bicyclo derivatives are described in the previous section as antifungal compounds [54,64]. Other VOCs identified in these experiments were

- **Linalool oxide**: This is a monoterpene acyclic tertiary alcohol that has been described as having antifungal activity against the fungus plant pathogen *Guignardia camelliae* [65], *Candida albicans* [66], and *Trichophyton rubrum* [67]. It has a lot of applications as a vitamin E precursor, in cosmetics, and in detergents, being the most widely used terpene in the food industry because of its fragrant and flavor properties [68]. This compound is also produced by many plants (*Coriandrum sativum L.*, *Cymbopogon martini* var 11artini, *Citrus sinensis* Osbeck, among others) as part of their essential oils [69].
- **Cis-5-ethenyltetrahydro-alpha, alpha, 5-trimethyl-2-Furanmethanol (linalool oxide B)**: This is a derivate of the previous molecule, present in the essential oils of several plants of the *Pittosporum* genus (among others), with cytotoxic, antimicrobial, and anti-inflammatory activities [70].
- **2-Methylenebornane**: This is a dehydrated form of 2-methylisoborneol produced by several microorganisms, especially Actinomycetes. This compound, together with

geosmin, is responsible for odors in water and soil. It has been reported that several antimicrobial activities are exerted by this compound [65,71].

All these VOCs might be involved in the inhibition of *E. weberi* growth. The analysis of volatile compounds produced both by CS131 confrontation on SFM and CS131 on SFM media (Table S4, Supplementary Material) also reveals the production of compounds with antimicrobial activity, such as trans-linalool oxide, whose activity depends on its stereochemical form [70]. In addition, different tetrasulfides with described antifungal activities [58] were detected.

Based on a bioinformatic analysis of the *Streptomyces* sp. CS131 genome [5], it can be seen that cluster 1.3 might be responsible for geosmin biosynthesis (100% similarity with the previously described cluster). Similarly, clusters 1.7 and 1.33a are involved in isorenieratene biosynthesis (87% and 100% similarity), and cluster 1.30 is involved in hopene biosynthesis (69% similarity). Clusters 1.9 (19% similarity with steffimycin biosynthesis cluster) and 1.23 (no similarity with any cluster described) have a low percentage of similarity with other described clusters, so they could be responsible for the synthesis of some of the terpenes identified using GS-MS in this strain since terpene cyclase/synthase genes have been identified in them (*ctg1_942* and *ctg1_5031*, respectively) and, therefore, in the topic of interest.

3.3.3. Cocultured *E.weberi–Streptomyces* sp. CS147

The assays confronting the *Streptomyces* sp. CS147 strain with *E. weberi* on SFM medium showed that a total of seventy-nine compounds were captured by the activated carbon and extracted with ethyl acetate; they were detected via GS-MS and identified using the NIST20 library (Table S5, Supplementary Material). From those, only five were exclusively produced and detected in the confrontation samples on SFM medium (Table S6, Supplementary Material). The other twenty-eight were also produced by other samples (Figure 6).

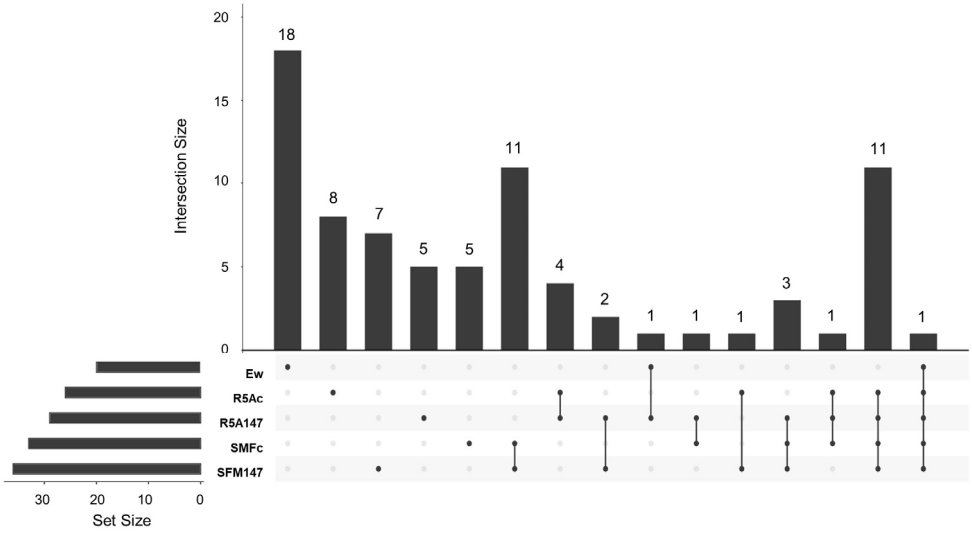

Figure 6. Upset Plot from the compounds detected via GS-MS from the assay confronting *Streptomyces* sp. CS147 and *Escovopsis weberi*. Ew: volatiles produced by *E. weberi*; SFMc: volatile produced during the coculture of *E. weberi–Streptomyces* CS147 on soy flour mannitol (SFM) medium; SFM147: volatiles produced by CS147 on SFM; R5A147: volatiles produced by CS147 on R5A medium; R5Ac: volatiles produced during the coculture of *E. weberi–Streptomyces* sp. CS147 on R5A.

About these five compounds were found exclusively during SFM medium confrontation, we observed that 3-octanone, previously described as having antifungal properties [54], was also detected in this strain. No antimicrobial activity has been described

for 3,5-Di-tert-butyl-2-hydroxybenzaldehyde in the literature. Concerning other VOC identifications, see the following:

- **5-diene-cis-Muurola-4(15)**: The derived compounds are components of plant essential oils that show antimicrobial properties [57,72,73].
- **2-methyl-nonadecane: This** has been described as being produced by *Streptomyces* sp. strains, and it is described as a molecule that contributes to the inhibition of phytopathogenic fungi. *Streptomyces* strain H3-2 has been sought to control Banana Fusarium Wilt because of the production of 2-methylnonadecane, among other volatile compounds [74].
- **1,3-dihydro-5-methoxy-2H-Benzimidazol-2-one:** No bioactivity has been described for this compound, but some derivatives of this molecule are related to antimicrobial activities. Agastache honey, which has 1,3-dihydro-5-methyl 2H-benzimidazol-2-one in its composition, has been described as presenting antifungal properties [75].

The analysis of compounds produced by *Streptomyces* sp. CS147 confrontation on SFM and CS147 on SFM also reveal the production of other compounds with antimicrobial activity: camphene, 1,3,3-trimethyl-tricyclo [2.2.1.0(2,6)] heptane, and 2-methylborname, whose properties have been described above. It is important to highlight the coincidence of many compounds between the assays of the different strains employed in this study. Dimethyl trisulfide and (1S)-6,6-dimethyl-2-methylene-bicyclo [3.1.1] heptane (2(10)-Pinene) have also been described as having antimicrobial activities [76,77]. 1,1,4,4-tetramethyl-2,5-dimethylene-cyclohexane is a volatile produced by *Streptomyces* sp. Y1-14 that has shown antifungal activity against several fungi [77,78]. Azulenone derivatives have been described as part of essential oils with antimicrobial and cytotoxic activities. A similar effect occurs in benzoxepines with antifungal properties [79].

No biological activity has been described for 1,3-bis(1-methylethyl)-1,3-cyclopentadiene, but its properties as an antioxidant and antifungal have been shown in some 1,3-cyclopentadiene derivatives [80]. Neither activity has been described for trans-9-methylene-3-oxabicyclo[5.3.0]decan-2-one. According to an AntiSMASH [55] analysis of the *Streptomyces* sp. CS147 genome [5], cluster 1.3 is predicted to be involved in geosmin synthesis (100% similarity) and 1.24 in hopene synthesis (69% similarity). Furthermore, cluster 1.23 seems to be implicated in 2-methylisoborneol biosynthesis (100% similarity with the described cluster). On the other hand, clusters 1.7 and 1.26a are predictably involved in isorenieratene biosynthesis (87% and 100% similarity, respectively). In contrast, cluster 1.8 (19% similarity with steffimycin D BGC) and cluster 1.19 (no similarity with any cluster described) show low similarity with the described clusters, but terpene cyclases/synthases have been identified *(ctg1_1003* and *ctg1_5179*, respectively), which suggest their involvement in terpene synthesis.

3.3.4. Cocultured *E. weberi–Streptomyces* sp. CS014

Assays confronting strain CS014 with *E. weberi* showed that there were fifty-five volatiles captured by the activated carbon and extracted with ethyl acetate, detected via GS-MS and identified using the NIST20 library (Table S7, Supplementary Material). Of these, only four were exclusively produced and detected in confrontation samples on SFM medium (Table S8, Supplementary Material). The other eighteen were also detected in other samples (Figure 7).

As with the other strains, bibliographic research was performed on the compounds produced exclusively by the confrontation on SFM medium and its properties. 3-octanone and benzoxepin derivatives have been previously mentioned as having antifungal properties [54,79]. 5-methyl dodecane is considered a modified molecule of dodecane and was present in the control samples. The fourth volatile corresponds to alpha-calacorene, a sesquiterpenoid and component of essential oils produced by *Teucrium montanum*, *Daniellia oliveri*, and *Leptoderris micrantha*, among other plants, which has also shown antimicrobial properties [81,82].

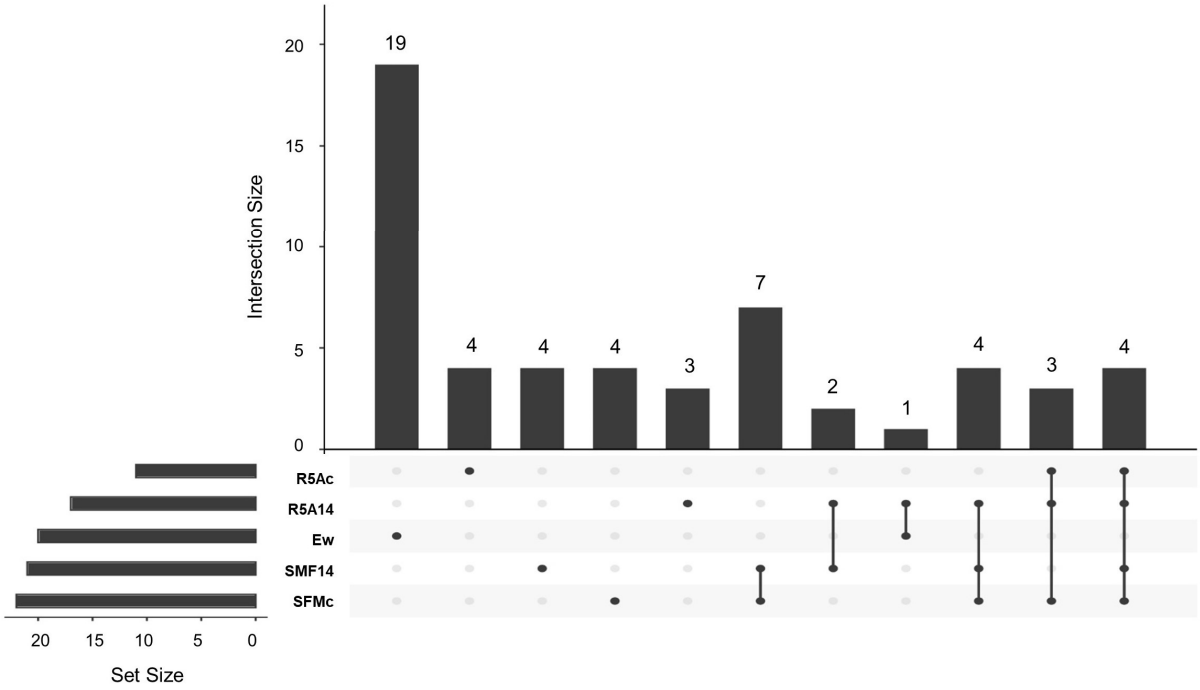

Figure 7. Upset Plot from the compounds detected via GS-MS from the assay confronting *Streptomyces* sp. CS014 and *Escovopsis weberi*. Ew: volatiles produced by *E. weberi*; SFMc: volatile produced during the coculture of *E. weberi–Streptomyces* sp. CS014 on soy flour mannitol (SFM) medium; SFM14: volatiles produced by CS014 on SFM; R5A14: volatiles produced by CS014 on R5A medium; R5Ac: volatiles produced during the coculture *E. weberi–Streptomyces* sp. CS014 on R5A.

The analysis of compounds produced by CS014 and CS014 confrontations on SFM also revealed the production of other compounds with antimicrobial activity: azulene derivatives such as 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl), [1R-(1.alpha.,3a.beta.,4.alpha.,7.beta.)]-azulene, and (S)-4,5,6,7,8,8a-hexahydro-8a-methyl-2(1H)-azulenone have been previously mentioned as components of essential oils with antifungal activities. The same applies to liguloxide, which is also part of plant essential oils [83]. (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene is a stereoisomer of cis-muurola-3,5-diene, which has already been described [59]. Previously, we revealed the antifungal properties of disulfite and furanone derivatives. However, no bioactivity has been described for 2-ethyl-heptane.

According to an AntiSMASH analysis of the *Streptomyces* sp. CS014 genome [5] regarding the identification of terpene biosynthesis gene clusters, cluster 1.4 is predicted to be involved in hopene synthesis (69% similarity), 1.20 is involved in isorenieratene biosynthesis (19% similarity), and 1.23 is involved in geosmin production (100% similarity). On the other hand, cluster 1.10 is also supposedly involved in the biosynthesis of terpene compounds because it has no similarity with any described cluster and has a terpene synthase/cyclase gene (*ctg1_1705*). This is the same for cluster 1.19 (19% similarity with steffimycin), which has the *ctg1_5927* gene.

It is worth noting that, of the four strains employed in this study, the predictions provided by AntiSMASH for terpene biosynthesis were very similar, which suggests similarities between microorganisms isolated from the same niche.

The analysis methodology used in this work to deal with VOCs allows for the extraction of the large number of volatile compounds retained in activated carbon [84]. However, it is important to note that this method has some limitations. On the one hand, there might be volatile compounds that are not retained in the activated charcoal, or they might volatilize when the recovery matrix is collected. Also, when the compounds are extracted with ethyl acetate, we can delimitate the analysis exclusively to those dissolved with that solvent. In addition, the GS-MS methodology presents a limit of detection in such a way that those compounds that present at low concentrations are not properly detected. Finally,

those that were not registered in the referenced library or that have not been described could not be identified and, consequently, they were discarded from the study. Thus, further analysis will be carried out to expand these studies.

It is worth mentioning that the numerous compounds that the strains employed produce are described as being part of the essential oils of different plants and used for different medicinal properties. Just as *Streptomyces* spp. produces various bioactive compounds that are also produced by plants [85], it makes sense that this phenomenon also occurs in volatile compounds.

All the results presented above suggest that various compounds might be responsible for the observed antifungal activity, mainly because of the abundant different VOCs identified in each analytical sample. It is important to highlight that the present work is not intended to assign antifungal activity to a single compound. The origin of a certain activity is usually linked to a particular compound or various compounds [86], not always in a causal relationship of 1:1. In these experiments, it can be seen how several compounds could jointly contribute to generating an environment hostile to fungal growth. The additive or synergistic effect between different volatiles results in a delay or inhibition of fungal growth. It seems straightforward that, for strains showing strong activity against *E. weberi*, antifungal compounds might normally be produced to a lesser extent, while a confrontation with *E. weberi* stimulates or increases the production of compounds that contribute to generating adverse conditions for fungal growth. It must also be taken into account that many compounds, such as (1S,4,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene in CS131, are not only found in samples of interest generated on SFM medium but are also produced by other strains grown on R5A medium. This means that such VOCs cannot be held responsible for antifungal activity, but neither can their collaboration in the final antifungal activity be denied. Despite all the descriptions of antifungal compounds made above, many of them have not been described individually as possessing these properties, but most of them are part of essential oil mixtures with antifungal activities without knowing if each one of their individual components has that particular effect, so it cannot be denied that some of them may have the aforementioned activity in and of themselves.

3.4. Dual Cultures against Other Fungi

In order to evaluate the antifungal effect on other fungi that are not pathogenic in the natural environment in which these *Streptomyces* spp. were isolated, an experiment was performed employing VOC chambers and using *Sclerotinia* spp. and *Phytophthora cinnamomi.* Both fungi are highly destructive plant pathogens [87,88]. *Sclerotinia* spp., like *E. weberi*, belong to the Ascomycota phylum, so their characteristics and life cycle are quite similar. Conversely, *P. cinnamomi* is an Oomycete, which implies more differences from the *E. weberi* pathogen.

The results showed that all of the *Streptomyces* sp. strains tested did not present antifungal activity against either of the two fungi (Figures 8 and 9). This fact represented an intriguing outcome since the production of volatile antifungal compounds by different *Streptomyces* strains is clearly not enough to prevent fungal growth in general terms. In addition, the results pointed to ecological implications supported by particular components that determine the observed effect manifesting solely against *E. weberi*, which is the natural pathogen in the environment where these *Streptomyces* strains were isolated. We cannot assume that the effect is exclusive and specific to *E. weberi*, but, at least, it is not effective on all types of fungi. Dhodary et al. [84] pointed out that this response could be a result of ammonia production by the different strains because *E. weberi* is highly sensitive to basification.

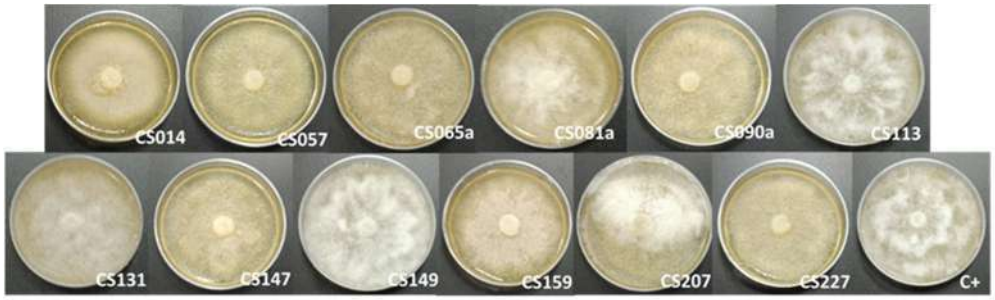

Figure 8. *Phytophthora cinnamomi* plates when cocultured in volatile organic compound (VOC) chambers against *Streptomyces* spp. CS014, CS57, CS065a, CS081a, CS090a, CS113 (first row), CS131, CS147, CS149, CS159, CS207, and CS227 (second row). The last image corresponds to the control of the assay (a VOC chamber without a *Streptomyces* sp. culture).

Figure 9. *Sclerotinia* spp. plates when cocultured in VOC (volatile organic compound) chambers against *Streptomyces* sp. CS014, CS57, CS065a, CS081a, CS090a, CS113 (first row), CS131, CS147, CS149, CS159, CS207, and CS227 (second row). The last image corresponds to the control of the assay (a VOC chamber without a *Streptomyces* sp. culture).

3.5. Analysis of Diffusible Compound Production

Culture samples of the different strains that showed antifungal effects on *E. weberi* were extracted with different organic solvents. This assay aimed to evaluate whether there were differences in the chromatographic profile of the *Streptomyces* spp. confronted with *E. weberi* in comparison with their counterparts confronted with *P. cinnamomi* (where no antifungal activity was detected) and those not confronted with any microorganism.

The results obtained showed that, in the conditions tested, there are some differences between the diffusible production profiles of *Streptomyces* spp. when confronted with *E. weberi* or *P. cinnamomi* and when there is no confrontation. In the case of strain CS057, the activation of skyllamycins A and B can be seen. In addition, in the CS014 activation of different granaticins, it can also be seen. Therefore, in terms of diffusible compound production, it is clear that the *Streptomyces* strains we tested react similarly to the near presence of both *E. weberi* and *P. cinnamomi* (Figure S3, Supplementary Material).

3.6. Multiple Culture

To evaluate if there is any synergistic antifungal effect when different *Streptomyces* spp. are present at the same time in a confined environment in the presence of *E. weberi*, three representatives of those microorganisms were cultured in small open dishes inside a large dish. In this way, we simulated an environment similar to that of a VOC chamber, where there is no physical contact between microorganisms or their diffusible compounds but there is an exchange of volatiles.

The results showed that certain combinations of isolated *Streptomyces* spp. increased their antifungal activity against *E. weberi*, a sum effect not observed with each individual strain alone. This effect is probably due to the saturation of antimicrobial compounds in the environment. Even though some *Streptomyces* spp. did not show antifungal activity in those assays that were previously shown (most probably because they do not produce VOCs in a

large enough quantity or because they produce few compounds with the required activity by accumulating both volatile productions together), it seems obvious that the joint effect provokes a manifest reduction in *E. weberi* growth (Figure 10).

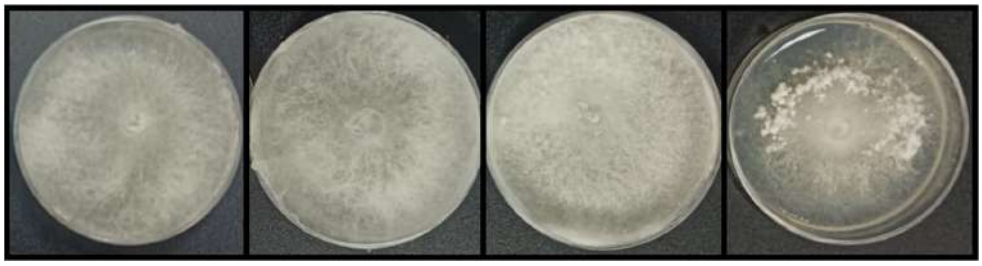

Figure 10. Reduction in *Escovopsis weberi* growth via multiple cocultures of different *Streptomyces* sp. strains. From left to right, *E. weberi* when cultured alone; *E. weberi* cultured in the presence of strain CS081a; *E. weberi* cultured in the presence of strain CS090a; *E. weberi* cultured in the presence of strains CS081a and CS090a.

3.7. pH Variation Analysis

It has been noted that the growth of *E. weberi* is inhibited by ammonia production, while *Leucoagaricus gongylophorus,* a fungus that lives in the environment of leafcutter ants, is not [84]. The role of this last fungus is very important for the ant ecosystem since it is in charge of degrading plant matter, thus serving as food for the ants [89].

To evaluate if the basification of the environment caused by volatile compounds can contribute to the inhibition of *E. weberi*, an assay with three small open plates inside a large one was performed: a plate containing *E. weberi*, a plate harboring a *Streptomyces* strain, and a plate of PDA supplemented with phenol red as pH indicator. Phenol red reagent is yellow at a pH below 6.8 and turns red when it is over 7.4. The results showed that all the strains that reveal antifungal activity against *E. weberi* make the indicator plate turn red at different levels, except for strain CS227 (Figure 11), whose color change is very slight.

Figure 11. Potato Dextrose Agar (PDA) + phenol red plates in VOC (volatile organic compound) chambers in the presence of *Streptomyces* spp. First row corresponds to plates cocultured with *Streptomyces* spp.; second row corresponds to plates cocultured with *Streptomyces* spp. and *Escovopsis weberi*.

These results suggest that the basification of an environment can contribute, together with the antifungal compounds previously described, to generating a hostile environment for pathogen development. In the case of CS227, it probably does not produce ammonia in a large enough quantity to provoke changes in the medium's pH. However, we do not assume that the inhibition is just induced by environment basification, but it might be one additional element that participates in the antifungal conditions generated. Supporting the previous assertion, strain CS081a has no volatile bioactivity against *E. weberi,* but it induces volatile basification in PDA plates (Figure 11). This fact highlights our hypothesis that the environmental basification of a medium can influence the antifungal bioactivity of these strains, but this is not the only factor.

It has been noted that in cocultures of *Streptomyces* spp. and yeast, once the nutrient source is depleted, the *Streptomyces* spp. are stimulated to produce trimethylamine (TMA), which not only alkalizes the environment but also favors its exploratory potential [6]. In the fight for space, nutrients, the colonization of an environment, and the chance to parasitize other organisms, microorganisms use all kinds of strategies to make their way into the

ecosystem, thus inducing a wide variety of responses from other competitors. These tools are essential for the correct maintenance of the environment, and their absence might cause an imbalance with a consequent domino effect on other members of that ecological niche or trophic chain.

We have shown that a combination of different elements with various natures and properties collaborate to inhibit pathogen development. It has previously been noted that volatile compounds can impede or slow entomopathogenic growth [90,91]. Although there are multiple reasons why bacteria produce volatile compounds (signaling, competition, etc.), it is plausible that the production of antifungal compounds could be because bacteria grow slower than fungi.

The present work shows the importance of VOCs in soil communities where different organisms might be dwelling in close proximity but at distances where the diffusible antimicrobial compounds do not have an action range [5]. The study of VOC emissions, typically performed using GS-MS, is difficult given the gaseous nature of the compounds and the ease of sample contamination from other volatiles present in the environment. In addition, the culture media not only determines the bacterial production of diffusible compounds but also produces volatile compounds [90,92]. However, with those limitations, it has been estimated that nearly 50–80% of bacteria produce volatile compounds under laboratory conditions [90], so it is worth focusing efforts on the discovery of new VOCs with recognized biological activities.

4. Conclusions

From the analysis of the antifungal activity of the twelve *Streptomyces* strains isolated from leafcutter ants, we found that seven of them showed antifungal activity against *E. weberi*, causing inhibition or a great delay in the fungal growth. The volatilomes of four of these strains were analyzed via GS-MS, revealing the presence of a large number of compounds, many of them described in the bibliography as antifungal or belonging to mixtures exerting such activity. These findings suggest that this inhibition could be due to the accumulation of various compounds with antimicrobial properties, excluding the option of assigning the observed activity to a specific compound. On the other hand, it has been estimated that the ammonium produced by these strains also contributes to generating an unfavorable environment for *E. weberi* growth, although that factor is not the only reason for the antifungal activity. In addition, this work reveals, once again, the multitude and complexity of the interactions that take place in ant nests and the bioactive potential of microorganisms isolated from this environment.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms11071820/s1: Figure S1: Device used on multiple cocultures of *Escovopsis weberi–Streptomyces* spp. Figure S2: Coculture of *Escovopsis weberi* and *Streptomyces* spp. on PDA, R5A, and SFM. Figure S3: Overview of chromatographic profile of *Streptomyces* sp. CS057 when cultured on SFM and extracted with butanol. Table S1: Compounds identified via GS-MS in the assay of volatile organic compounds produced by the confrontation of *Streptomyces* sp. CS057 and *E. weberi*. Table S2: Summary of compounds identified via GS-MS in the assay of volatile organic compounds produced by the confrontation of *Streptomyces* sp. CS057 and *E. weberi.* Table S3: Compounds identified via GS-MS in the assay of volatile organic compounds produced by the confrontation of *Streptomyces* sp. CS131 and *E. weberi.* Table S4: Summary of compounds identified via GS-MS in the assay of volatile organic compounds produced by the confrontation of *Streptomyces* sp. CS131 and *E. weberi.* Table S5: Compounds identified via GS-MS in the assay of volatile organic compounds produced by the confrontation of *Streptomyces* sp. CS147 and *E. weberi*. Table S6: Summary of compounds identified via GS-MS in the assay of volatile organic compounds produced by the confrontation of *Streptomyces* sp. CS147 and *E. weberi*. Table S7: Compounds identified via GS-MS in the assay of volatile organic compounds produced by the confrontation of *Streptomyces* sp. CS014 and *E. weberi*. Table S8: Summary of compounds identified via GS-MS in the assay of volatile organic compounds produced by the confrontation of *Streptomyces* sp. CS014 and *E. weberi.*

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Supplementary data

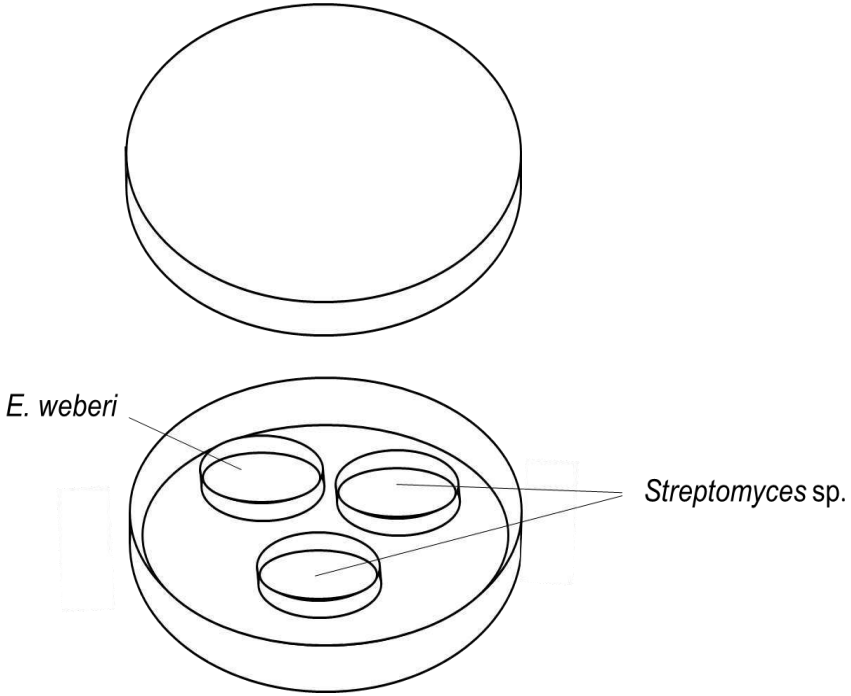

Figure S1: Device used on multiple coculture *E. weberi*-*Streptomyces* spp.

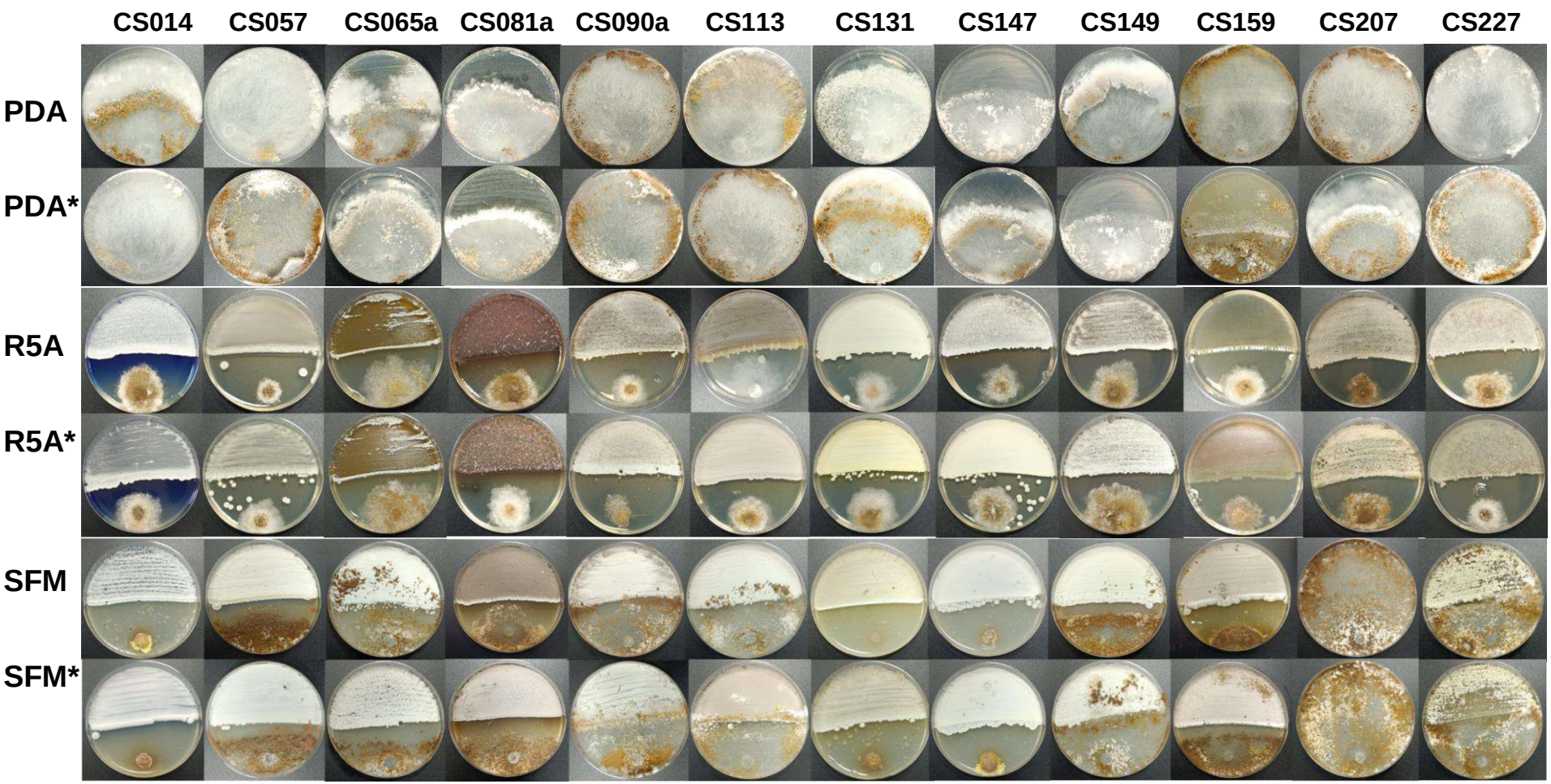

Figure S2: Coculture of *Escovopsis weberi* and *Streptomyces* spp. on Potato Dextrose Agar (PDA), R5A, and Soy Flour Mannitol (SFM). The asterisk indicates the confrontation experiment using two days-grown *Streptomyces* spp.

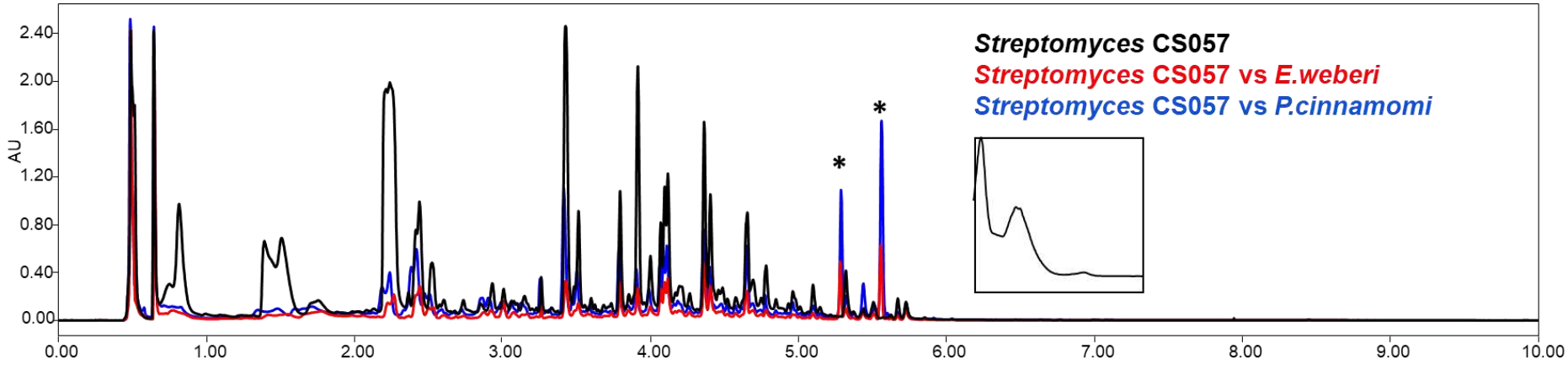

Figure S3: Overview of chromatographic profile of *Streptomyces* sp. CS057 cultured in Soy Flour Mannitol (SFM) medium and extracted with butanol. Activation of skyllamycins A and B biosynthesis can be observed (asterisks) by the presence of fungi *Escovopsis weberi* or *Phytophthora cinnamomi*. UV-Vis spectrum of skyllamycins is shown.

Table S1. Compounds identified by GS-MS in the assay of Volatile Organic Compounds produced by the confrontation *Streptomyces* CS057 and *E. weberi* (Ew: volatiles produced by *E. weberi*; SFM-confrontation: volatiles produced during the confrontation *E.weberi* - *Streptomyces* CS057 on SFM; SFM-CS057: volatiles produced by CS57 on SFM; R5A-CS057: volatiles produced by CS057 on R5A; R5A-confrontation: volatiles produced during the confrontation *E.weberi* - *Streptomyces* CS057).

SFM-CS057	SFM-confrontation	R5A-CS057	R5A-confrontation	Ew
• Naphthalene, 1,2,3,4-tetrahydro-1,6-dimethyl-4-(1-methylethyl)-, (1S-cis) • 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)] • trans-1,10-Dimethyl-trans-9-decalinol • 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl • S-Methylmethanethiosulphonate • p-Menth-8-ene, 3-methylene- • 1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)-	• Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl • Camphene • Dimethyltrisulfide • Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)- • 3-Octanone • p-Menth-8-ene, 3-methylene • 3,6-Heptanedione • Octanoic acid, 2-propenyl ester • 4,8-dimethylnona-1,3,7-triene • 2,3-Dimethyl-5-oxohexanethioic acid, S-t-butylester • 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, trans	• Pyrazine, 2,5-dimethyl- • Dimethyltrisulfide • Benzene, 1-ethoxy-4-ethyl- • p-Menth-8-ene, 3-methylene- • S-Methylmethanethiosulphonate • Vinyl 2-ethylhexanoate • 2,3-Dimethyl-5-oxohexanethioic acid, S-t-butylester • Bromoacetylbromide • 2-Methylisoborneol • Allylnonanoate • trans-1,10-Dimethyl-trans-9-decalinol	• 3,5-Di-tert-butyl-2-hydroxybenzaldehyde • cis-Calamenene • Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis) • D-Alanine, N-(4-butylbenzoyl)-, heptylester • 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro--1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]- • trans-1,10-Dimethyl-trans-9-decalinol • 2,4,4,6,6,8,8-Heptamethyl-2-nonene	• 2,6-Di-tert-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one • Isophthalicacid, 3,5-difluorophenyl heptylester • Phosphoramidousdifluoride, dimethyl • 3-Butene-1,2-diol • 1-Dodecanol • Bicyclo[3.2.0]heptan-2-one, 6-hydroxy-5-methyl-6-vinyl • Succinimide • Pentanoicacid, 5-hydroxy-, 2,4-di-t-butylphenyl esters • 2-Methylisoborneol • Heptadecane

Table S1. Cont.

SFM-CS057	SFM-confrontation	R5A-CS057	R5A-confrontation	Ew
• Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)- • Dimethyltrisulfide • 2,3-Dimethyl-5-oxohexanethioic acid, S-t-butylester • Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl • 3-Octanone • 3,6-Heptanedione • 4,8-dimethylnona-1,3,7-triene • 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, trans • Aceticacid, trifloro-,1-methylpropyl ester • 2-Methylisoborneol	• 2-Methylisoborneol • 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl • trans-1,10-Dimethyl-trans-9-decalinol • 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)] • Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis) • cis-Calamenene	• 1H-Cyclopropa[a]naphthalene, -1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)] • Bicyclosesquiphellandrene • Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)- • cis-Calamenene • Tris(3-phenyl-2,4-pentanedionato)aluminum(iii)	• 1,5-Dioxaspiro[5.5]undecane, 3,3-dimethyl • Allylnonanoate • 2-Methylisoborneol • 2,3-Dimethyl-5-oxohexanethioic acid, S-t-butylester • S-Methylmethanethiosulphonate • p-Menth-8-ene, 3-methylene • Benzene, 1-ethoxy-4-ethyl • Dimethyltrisulfide	• 2,4,4,6,6,8,8-Heptamethyl-2-nonene • Pyrrolidine • Pyrazine, 2,5-dimethyl • Acetophenone • 2-Pentene, 2,4,4-trimethyl • 3-Heptene, 2,2,4,6,6-pentamethyl • Cyclohexane, 1-ethyl-1-methyl • Hexane, 3,3-dimethyl • Bis(2-isopropyl-5-methylcyclohexyl) methylphosphonate (isomer 2) • 3,6-Dimethylpiperazine-2,5-dione

Table S2. Summary of compounds identified by GS-MS in the assay of Volatile Organic Compounds produced by the confrontation of *Streptomyces* CS057 and *E. weberi.* In the first column: VOCs exclusively detected on SFM confrontation; in the second, VOCs in common between SFM confrontation and SFM57.

VOCs exclusively in CS057 SFM-confrontation	VOCs in common in SFM-confrontation and SFM-CS057
• Camphene • Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)- • Octanoic acid, 2-propenyl ester	• 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl • Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl • 3-Octanone • 3,6-Heptanedione • 4,8-dimethylnona-1,3,7-triene • 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, trans

Table S3. Compounds identified by GS-MS in the assay of Volatile Organic Compounds produced by the confrontation *Streptomyces* CS131 and *E. weberi* (Ew: volatiles produced by *E. weberi*; SFM-confrontation: volatiles produced during the confrontation *E.weberi* - *Streptomyces* CS131 on SFM; SFM-CS131: volatiles produced by CS131 on SFM; R5A-CS131: volatiles produced by CS131 on R5A; R5A-confrontation: volatiles produced during the confrontation *E.weberi* - *Streptomyces* CS131.

SFM-CS131	SFM-confrontation	R5A-CS131	R5A-confrontation	Ew
• 2(3H)-Furanone, dihydro-3-methyl-	• Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	• Pyrazine, 2,5-dimethyl-	• 2(3H)-Furanone, dihydro-3-methyl-	• 2,6-Di-tert-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one
• Dimethyltrisulfide	• 2(3H)-Furanone, dihydro-3-methyl-	• 2(3H)-Furanone, dihydro-3-methyl-	• 1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)-	• Isophthalicacid, 3,5-difluorophenyl heptylester
• 1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)-	• Dimethyltrisulfide	• Dimethyltrisulfide	• 2H-Pyran-2-one, tetrahydro-	• Phosphoramidousdifluoride, dimethyl
• p-Menth-8-ene, 3-methylene-	• Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	• 1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)-	• Dimethyltrisulfide	• 3-Butene-1,2-diol
• trans-Linalool oxide (furanoid)	• 1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)-	• p-Menth-8-ene, 3-methylene-	• Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	• 1-Dodecanol
• 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, trans-	• 2-Methylenebornane	• 1,7,7-Trimethylbicyclo[2.2.1]hept-5-en-2-one	• 3-Heptene, 2,2,4,6,6-pentamethyl-	• Bicyclo[3.2.0]heptan-2-one, 6-hydroxy-5-methyl-6-vinyl
• 2-Methylisoborneol	• 3-Octanone	• 2-Methylisoborneol	• p-Menth-8-ene, 3-methylene-	
• Tetrasulfide, dimethyl	• 3-Heptene, 2,2,4,6,6-pentamethyl-		• Cyclopentane, 1,1,3,4-tetramethyl-, cis-	

Table S3. Cont.

SFM-CS131	SFM-confrontation	R5A-CS131	R5A-confrontation	Ew
• 2,6-Dimethyl-2-trans-6-octadiene	• Disulfide, bis(1,1,3,3-tetramethylbutyl)	• Fumaricacid, decyl 2-methylcyclohex-1-enylmethyl ester	• Pentane, 3,3-diethyl-	• Succinimide
• 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-	• p-Menth-8-ene, 3-methylene-	• 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-	• 4,8-dimethylnona-1,3,7-triene	• Pentanoicacid, 5-hydroxy-, 2,4-di-t-butylphenyl esters
• Cyclohexanone, 2,5-dimethyl-2-(1-methylethenyl)-	• 2-Furanmethanol, 5-ethenyltetrahydro-.alpha.,.alpha.,5-trimethyl-, cis-	• Cyclohexanone, 2,5-dimethyl-2-(1-methylethenyl)-1,5-Heptadien-4-ol, 3,3,6-trimethyl-	• 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, trans-	• 2-Methylisoborneol
• 2-Undecanone, 6,10-dimethyl-trans-1,10-Dimethyl-trans-9-decalinol	• trans-Linalool oxide (furanoid)	• Cyclopentane, 1,1,3,4-tetramethyl-, cis-	• 2-Methylisoborneol	• Heptadecane
• 9-Undecenal, 2,10-dimethyl-	• Pentane, 3,3-diethyl-	• 2-Undecanone, 6,10-dimethyl-	• 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-	• 2,4,4,6,6,8,8-Heptamethyl-2-nonene
• 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]-	• 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, trans-	• trans-1,10-Dimethyl-trans-9-decalinol	• Cyclohexanone, 2,5-dimethyl-2-(1-methylethenyl)-	• Pyrrolidine
	• 2-Methylisoborneol	• 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-	• 2,4,4,6,6,8,8-Heptamethyl-2-nonene	• Pyrazine, 2,5-dimethyl
	• Tetrasulfide, dimethyl		• 1,5-Heptadien-4-ol, 3,3,6-trimethyl-	
	• 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-			

Table S3. Cont.

SFM-CS131	SFM-confrontation	R5A-CS131	R5A-confrontation	Ew
• 1-Isopropyl-4,7-dimethyl-1,2,3,4,5,6-hexahydronaphthalene • cis-Muurola-4(15),5-diene • cis-Calamenene • Caryophyllenyl alcohol • 4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S,4S,4aS,8aR)-	• Cyclohexanone, 2,5-dimethyl-2-(1-methylethenyl)- • 1,2,4,5-Tetrazin-3-amine • 2-Undecanone, 6,10-dimethyl- • trans-1,10-Dimethyl-trans-9-decalinol • (1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene • 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]- • (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene	• 1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]- • (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene • cis-Muurola-4(15),5-diene • Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1.alpha.,4a.alpha.,8a.alpha.)- • Hexane, 3,3-dimethyl- • cis-Calamenene • Naphthalene, 1,2,3,4,4a,7-hexahydro-1,6-dimethyl-4-(1-methylethyl)- • Caryophyllenyl alcohol	• (1S,2E,6E,10R)-3,7,11,11-Tetramethylbicyclo[8.1.0]undeca-2,6-diene • 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]- • 2-Undecanone, 6,10-dimethyl- • cis-Calamenene • Caryophyllenyl alcohol • 1-Undecene, 7-methyl- • 4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S,4S,4aS,8aR)-	• Acetophenone • 2-Pentene, 2,4,4-trimethyl • 3-Heptene, 2,2,4,6,6-pentamethyl • Cyclohexane, 1-ethyl-1-methyl • Hexane, 3,3-dimethyl • Bis(2-isopropyl-5-methylcyclohexyl) methylphosphonate (isomer 2) • 3,6-Dimethylpiperazine-2,5-dione

Table S3. Cont.

SFM-CS131	SFM-confrontation	R5A-CS131	R5A-confrontation	Ew
	• Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis) • 1-Isopropyl-4,7-dimethyl-1,2,3,4,5,6-hexahydronaphthalene • Bicyclosesquiphellandren • cis-Muurola-4(15),5-diene • cis-Calamenene • Caryophyllenyl alcohol • cis-Linaloloxide • 4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S,4S,4aS,8aR)-	• 4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S,4S,4aS,8aR)-	• (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene • Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)- • 1-Isopropyl-4,7-dimethyl-1,2,3,4,5,6-hexahydronaphthalene • Bicyclosesquiphellandrene	

Table S4. Summary of compounds identified by GS-MS in the assay of Volatile Organic Compounds produced by the confrontation of *Streptomyces* CS131 and *E. weberi*. In the first column, VOCs exclusively detected on SFM confrontation; in the second, VOCs in common between SFM confrontation and SFM131

VOCs exclusively in CS131 SFM-confrontation	VOCs in common in SFM-confrontation and SFM-CS131
• 1,2,4,5-Tetrazin-3-amine • cis-Linalool oxide • Disulfide, bis(1,1,3,3-tetramethylbutyl) • 2-Furanmethanol, 5-ethenyltetrahydro-alpha, alpha,5-trimethyl-, cis- • 2-Methylenebornane • 3-Octanone • Bicyclo[3.1.0]hex-2-ene, 2-methyl-5-(1-methylethyl)-	• trans-Linalool oxide (furanoid) • Tetrasulfide, dimethyl

Table S5. Compounds identified by GS-MS in the assay of Volatile Organic Compounds produced by the confrontation *Streptomyces* CS147 and *E. weberi* (Ew: volatiles produced by *E. weberi*; SFM-confrontation: volatiles produced during the confrontation *E.weberi* - *Streptomyces* CS147 on SFM; SFM-CS147: volatiles produced by CS147 on SFM; R5A-CS147: volatiles produced by CS147 on R5A; R5A-confrontation: volatiles produced during the confrontation *E.weberi* - *Streptomyces* CS147.

SFM-CS147	SFM-confrontation	R5A-CS147	R5A-confrontation	Ew
• Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-	• Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-	• Benzene, 1-ethoxy-4-ethyl-	• Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	• 2,6-Di-tert-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one
• Camphene	• Camphene	• Bicyclo[3.1.0]hex-2-ene, 4 -methyl-1-(1-methylethyl)-	• Octane, 2-methyl-	• Isophthalicacid, 3,5-difluorophenyl heptylester
• 1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)-	• 1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)-	• p-Menth-8-ene, 3-methylene-	• Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-	• Phosphoramidousdifluoride, dimethyl
• Dimethyl trisulfide	• Dimethyltrisulfide	• Decane, 4-methyl-	• Benzene, 1-ethoxy-4-ethyl-	• 3-Butene-1,2-diol
• Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	• 3-Octanone	• 4,8-dimethylnona-1,3,7-triene	• p-Menth-8-ene, 3-methylene-	• 1-Dodecanol
• Bicyclo[3.1.1]heptane, 6,6-dime thyl-2-methylene-, (1S)-	• Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	• 1,7,7-Trimethylbicyclo[2.2.1]hept-5-en-2-one	• 4,8-dimethylnona-1,3,7-triene	• Bicyclo[3.2.0]heptan-2-one, 6-hydroxy-5-methyl-6-vinyl
• 2-Methylenebornane	• Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)-	• 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, cis-	• 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, cis-	• Succinimide
• p-Menth-8-ene, 3-methylene-	• p-menth-8-ene, 3-methylene-	• 2-Methylisoborneol		

Table S5. Cont.

SFM-CS147	SFM-confrontation	R5A-CS147	R5A-confrontation	Ew
• Decane, 4-methyl-	• 4,8-dimethylnona-1,3,7-triene	• 1H-Indene, 1-ethylideneoctahydro-7a-methyl-, (1Z,3a.alpha.,7a.beta.)-	• 2-Methylisoborneol	• Pentanoicacid, 5-hydroxy-, 2,4-di-t-butylphenyl esters
• 4,8-dimethylnona-1,3,7-triene	• 2-Methylenebornane	• 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-	• 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-	• 2-Methylisoborneol
• Octane, 5-ethyl-2-methyl-	• 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, cis-	• Tricyclo[3.3.3.0]undecan-3-one	• Cyclohexanone, 2,5-dimethyl-2-(1-methylethenyl)-	• Heptadecane
• Ethanone, 1-[2-methyl-5-(1-methylethenyl)cyclopentyl]-, (1.alpha.,2.alpha.,5.beta.)-	• 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, trans-5-(1-methylvinyl)-8-methyl-	• 2-Caren-4-ol	• Tricyclo[3.3.3.0]undecan-3-one	• 2,4,4,6,6,8,8-Heptamethyl-2-nonene
• 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, cis-		• 2,4,4,6,6,8,8-Heptamethyl2 -2-nonene		
• 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, trans-	• Liguloxide	• trans-1,10-Dimethyl-trans-9-decalinol	• Decane, 2,3,6-trimethyl-	• Pyrrolidine
• 2-Methylisoborneol	• 1-Isopropyl-4,7-dimethyl-1,2,3,4,5,6-hexahydronaphthalene	• 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]-	• Silane, diethylhexyloxytridecyloxy	• Pyrazine, 2,5-dimethyl
• 1H-Benzocycloheptene, 4,4a,5,6,7, 8,9,9a-octahydro-4a-methyl-, trans-	• cis-Muurola-4(15),5-diene		• cis-Chrysanthenol	• Acetophenone
	• Bicyclosesquiphellandrene			

Table S5. Cont.

SFM-CS147	SFM-confrontation	R5A-CS147	R5A-confrontation	Ew
• 1H-Indene, 1-ethylideneoctahydro-7a-methyl-, (1Z,3a.alpha.,7a.beta.)-	• 2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha.)]-	• (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene	• trans-1,10-Dimethyl-trans-9-decalinol	• 2-Pentene, 2,4,4-trimethyl
• Cyclohexane, 1,1,4,4-tetramethyl-2, 5-dimethylene-	• Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	• Naphthalene, 1,2,3,5,6,7, 8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [1R-(1 alpha.,7.beta.,8a.alpha.)]-	• 1H-Cyclopropa[a]naphthalene, 1a, 2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]-	• 3-Heptene, 2,2,4,6,6-pentamethyl
• 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-	• cis-Calamenene	• Liguloxide	• Liguloxide	• Cyclohexane, 1-ethyl-1-methyl
• Propanoic acid, 2,2-dimethyl-, anhydride with diethylborinic acid	• 2H-Benzimidazol-2-one, 1,3-dihydro-5-methoxy-	• 1R,2S,6S,7S,8S)-8-Isopropyl(-1-methyl-3-methylenetricyclo[4.4.0.02,7]decane-rel-	• Bicyclosesquiphellandrene	• Hexane, 3,3-dimethyl
• 2(1H)-Azulenone, 4,5,6,7,8,8a-hexahydro-8a-methyl-, (S)-	• Nonadecane, 2-methyl-	• 2-Bromotetradecane	• Caryophyllenyl alcohol	• Bis(2-isopropyl-5-methylcyclohexyl) methylphosphonate (isomer 2)
• Nonane, 5-(2-methylpropyl)-	• 2-Methylisoborneol	• Bicyclosesquiphellandrene	• cis-Calamenene	• 3,6-Dimethylpiperazine-2,5-dione
• trans-1,10-Dimethyl-trans-9-decalinol	• 1H-Indene, 1-ethylideneoctahydro-7a-methyl-, (1Z,3a.alpha.,7a.beta.)-	• 1-Isopropyl-4,7-dimethyl1,2,3,4,5,6-hexahydronaphthalene-	• Decane, 3-ethyl-3-methyl-	

Table S5. Cont.

SFM-CS147	SFM-confrontation	R5A-CS147	R5A-confrontation	Ew
• 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]-	• Cyclohexane, 1,1,4,4-tetramethyl-2,5-dimethylene-	• Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	• 4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S,4S,4aS,8aR)-	
• Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,3a.beta.,4.alpha.,7.beta.)]-	• 1-Cyclohexene-1-acetaldehyde, 2,6,6-trimethyl-	• cis-Calamenene		
• (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene	• 2(1H)-Azulenone, 4,5,6,7,8, 8a-hexahydro-8a-methyl-, (S)-	• Caryophyllenyl alcohol	• 2,7-Cyclodecadiene-1-methanol, alpha.,.alpha.,4,8-tetramethyl-	
• Bicyclo[5.3.0]decane, 2-methylene-5-(1-methylvinyl)-8-methyl-	• trans-1,10-Dimethyl-trans-9-decalinol	• 4a(2H)-Naphthalenol, 1,3,4, 5,6,8a-hexahydro-4,7-dimet hyl-1-(1-methylethyl)-, (1S,4S,4aS,8aR)-	• Isophthalic acid, ethyl tridec-2-ynyl ester	
• Liguloxide	• 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]-	• 2,7-Cyclodecadiene-1-methanol, .alpha.,.alpha.,4,8-tetramethyl-	• 1-Benzyloxy-1-ethyl-1-silacyclopentane	
• 2-Bromotetradecane	• (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene			
• Bicyclosesquiphellandrene	• Bicyclo[5.3.0]decane, 2-methylene-2,7-Cyclodecadiene-1-methanol, .alpha.,.alpha.,4,8-tetramethyl-	• 1-Benzyloxy-1-ethyl-1-silacyclopentane	• Isophthalic acid, di(2-fluorophenyl) ester	

Table S5. Cont.

SFM-CS147	SFM-confrontation	R5A-CS147	R5A-confrontation	Ew
• 2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha.)]- • cis-Calamenene • Naphthalene, 1,2,3,5,6,8a-hexahydro • 4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S,4S,4aS,8aR)- • 4H-Benzo[b]pyrane-3-carbonitrile, 5,6,7,8-tetrahydro-2-amino-4-(3-cyclohexenyl)-7,7-dimethyl-5-oxo • Isophthalic acid, di(2-fluorophenyl) ester	• 4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S,4S,4aS,8aR)- • 3,5-Di-tert-butyl-2-hydroxybenzaldehyde • 2,7-Cyclodecadiene-1-methanol, .alpha.,.alpha.,4,8-tetramethyl-	• 2-Amino-4-(2-cyclohexyl-ethyl)-7		

Table S6. Summary of compounds identified by GS-MS in the assay of Volatile Organic Compounds produced by the confrontation of *Streptomyces* CS147 and *E. weberi.* In the first column, VOCs exclusively detected on SFM confrontation, in the second; VOCs in common between SFM confrontation and SFM147

VOCs exclusively in CS147 SFM-confrontation	VOCs in common in SFM-confrontation and SFM-CS147
• 3-Octanone • cis-Muurola-4(15),5-diene • 3,5-Di-tert-butyl-2-hydroxybenzaldehyde • Nonadecane, 2-methyl- • 2H-Benzimidazol-2-one, 1,3-dihydro-5-methoxy-	• Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl- • Camphene • 1,3-Cyclopentadiene, 1,3-bis(1-methylethyl)- • Dimethyl trisulfide • Bicyclo[3.1.1]heptane, 6,6-dimethyl-2-methylene-, (1S)- • 2-Methylenebornane • 3-Oxabicyclo[5.3.0]decan-2-one, 9-methylene-, trans- • Cyclohexane, 1,1,4,4-tetramethyl-2,5-dimethylene- • 2(1H)-Azulenone, 4,5,6,7,8,8a-hexahydro-8a-methyl-, (S)- • Bicyclo[5.3.0]decane, 2-methylene-5-(1-methylvinyl)-8-methyl- • 2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha.)]-

Table S7. Compounds identified by GS-MS in the assay of Volatile Organic Compounds produced by the confrontation *Streptomyces* CS014 and *E. weberi* (Ew: volatiles produced by *E. weberi*; SFM-confrontation: volatiles produced during the confrontation *E.weberi* - *Streptomyces* CS014 on SFM; SFM-CS014: volatiles produced by CS14 on SFM; R5A-CS014: volatiles produced by CS014 on R5A; R5A-confrontation: volatiles produced during the confrontation *E.weberi* - *Streptomyces* CS014.

SFM-CS014	SFM-confrontation	R5A-CS014	R5A-confrontation	Ew
• 2(3H)-Furanone, dihydro-3-methyl- • Dimethyl trisulfide • Heptane, 4-ethyl- • Decane, 2,4-dimethyl- • Tetrasulfide, dimethyl • 1H-Indene, 1-ethylideneoctahydro-7a-methyl-, (1Z,3a.alpha.,7a.beta.)- • Cyclohexane, 1,1,4,4-tetramethyl-2,5-dimethylene-	• alpha-Calacorene • Cubenene • cis-Calamenene • Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)- • 2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha.)]- • .alpha.-Muurolene • cis-Muurola-4(15),5-diene	• Dimethyl trisulfide • Bicyclo[4.3.0]non-3-ene, 3,4,7-trimethyl- • 1H-Indene, 1-ethylideneoctahydro-7a-methyl-, (1Z,3a.alpha.,7a.beta.)- • Cyclohexane, 1,1,4,4-tetramethyl-2,5-dimethylene- • Cyclohexane, 1,1,4,4-tetramethyl-2,6-bis(methylene)- • 2,4,4,6,6,8,8-Heptamethyl-2-nonene	• 1H-Indene, 1-ethylideneoctahydro-7a-methyl-, (1Z,3a.alpha.,7a.beta.)- • Decane, 2,3,5-trimethyl- • Decane, 1-iodo- • 1-Tetradecene • 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]- • trans-1,10-Dimethyl-trans-9-decalinol	• 2,6-Di-tert-butyl-4-hydroxy-4-methylcyclohexa-2,5-dien-1-one • Isophthalicacid, 3,5-difluorophenyl heptylester • Phosphoramidousdifluoride, dimethyl • 3-Butene-1,2-diol • 1-Dodecanol • Bicyclo[3.2.0]heptan-2-one, 6-hydroxy-5-methyl-6-vinyl • Succinimide

Table S7. Cont.

SFM-CS014	SFM-confrontation	R5A-CS014	R5A-confrontation	Ew
• Bicyclo[4.3.0]non-3-ene, 3,4,7-trimethyl • Octane, 1-iodo- • 2(1H)-Azulenone, 4,5,6,7,8,8a-hexahydro-8a-methyl-, (S)- • Cyclopropanebutanoic acid, 2,4-dioxo-, methyl ester • Cubenene • trans-1,10-Dimethyl-trans-9-decalinol • Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,3a.beta.,4.alpha.,7.beta.)] • 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]-	• 1-Isopropyl-4,7-dimethyl-1,2,3,4,5,6-hexahydronaphthalene • Liguloxide • (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene • 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a- tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]- • Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,3a.beta.,4.alpha.,7.beta.)]- • trans-1,10-Dimethyl-trans-9-decalinol • Dodecane, 5-methyl-	• trans-1,10-Dimethyl-trans-9-decalinol • (-)-Aristolene • 1H-Cyclopropa[a]naphthalene, 1a,2,3,5,6,7,7a,7b-octahydro-1,1,7,7a-tetramethyl-, [1aR-(1a.alpha.,7.alpha.,7a.alpha.,7b.alpha.)]- • 1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z- • 1-Isopropyl-4,7-dimethyl-1,2,3,4,5,6-hexahydronaphthalene • cis-Muurola-4(15),5-diene • .alpha.-Muurolene	• 5-Methyl-2,4-diisopropylphenol • 1-Isopropyl-4,7-dimethyl-1,2,3,4,5,6-hexahydronaphthalene • .alpha.-Muurolene • cis-Calamenene • Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	• Pentanoicacid, 5-hydroxy-, 2,4-di-t-butylphenyl esters • 2-Methylisoborneol • Heptadecane • 2,4,4,6,6,8,8-Heptamethyl-2-nonene • Pyrrolidine • Pyrazine, 2,5-dimethyl • Acetophenone

Table S7. Cont.

SFM-CS014	SFM-confrontation	R5A-CS014	R5A-confrontation	Ew
• (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene • Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)- • Liguloxide • cis-Muurola-4(15),5-diene • Methyl (2R,3R,4S)-3-(tert-butyldimethylsilyloxy)-2,4-dimethylhexanoate • Octahydroxanthen-1,9-dione, 3, 3,7,7,-tetramethyl-10-hexyl-	• 2(1H)-Azulenone, 4,5,6,7,8,8a-hexahydro-8a-methyl-, (S)- • Cyclohexane, 1,1,4,4-tetramethyl-2,5-dimethylene- • 1H-Indene, 1-ethylideneoctahydro-7a-methyl-, (1Z,3a.alpha.,7a.beta.)- • Tetrasulfide, dimethyl • Heptane, 4-ethyl- • 3-Octanone • Dimethyl trisulfide • 2(3H)-Furanone, dihydro-3-methyl	• Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)- • cis-Calamenene • Cubenene • Methyl (2R,3R,4S)-3-(tert-butyldimethylsilyloxy)-2,4-dimethylhexanoate		• 2-Pentene, 2,4,4-trimethyl • 3-Heptene, 2,2,4,6,6-pentamethyl • Cyclohexane, 1-ethyl-1-methyl • Hexane, 3,3-dimethyl • Bis(2-isopropyl-5-methylcyclohexyl) methylphosphonate (isomer 2) • 3,6-Dimethylpiperazine-2,5-dione

Table S8. Summary of compounds identified by GS-MS in the assay of Volatile Organic Compounds produced by the confrontation of *Streptomyces* CS014 and *E. weberi*. In the first column, VOCs exclusively detected on SFM confrontation; in the second, VOCs in common between SFM confrontation and SFM14

VOCs exclusively in CS014 SFM-confrontation	VOCs in common in SFM-confrontation and SFM-CS014
• alpha-Calacorene • 2H-3,9a-Methano-1-benzoxepin, octahydro-2,2,5a,9-tetramethyl-, [3R-(3.alpha.,5a.alpha.,9.alpha.,9a.alpha.)]- • Dodecane, 5-methyl- • 3-Octanone	• Liguloxide • (1S,4S,4aS)-1-Isopropyl-4,7-dimethyl-1,2,3,4,4a,5-hexahydronaphthalene • Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1.alpha.,3a.beta.,4.alpha.,7.beta.)]- • 2(1H)-Azulenone, 4,5,6,7,8,8a-hexahydro-8a-methyl-, (S)- • Tetrasulfide, dimethyl • Heptane, 4-ethyl- • 2(3H)-Furanone, dihydro-3-methyl-

IV. Discusión

El incremento de la aparición de patógenos multirresistentes, así como de enfermedades emergentes, son las principales causas que reclaman nuevas estrategias para la detección de productos naturales desconocidos (Miethke *et al*., 2021; Serral *et al*., 2021). Ante esta finalidad, se precisan de aproximaciones innovadoras y creativas, dado que el proceso de descubrimiento se encuentra estancado debido al continuo reaislamiento de metabolitos ya conocidos, y la limitación que presentan ciertas técnicas de análisis (Atanasov *et al*., 2021). Son necesarios compuestos naturales novedosos para su empleo como materias primas, así como para generar derivados semisintéticos o análogos sintéticos con diferentes bioactividades (Pham *et al*., 2019; Stojković *et al*., 2023). Además, de la aplicación farmacéutica de los productos naturales como alternativas terapéuticas, estos también presentan interés en otros campos de la industria, los cuales también se pueden beneficiar de la caracterización de metabolitos novedosos.

El filo Actinobacteria constituye uno de los grupos más diversos en la naturaleza, con representantes con variedad de morfologías, muchas de ellas con ciclos de vida complejos y ampliamente distribuidas tanto en ambientes acuáticos como terrestres. Sin duda, el género *Streptomyces* es el miembro más importante de esta categoría, ya que genéticamente es un gran reservorio de compuestos naturales con propiedades bioactivas (Barka *et al*., 2016). Su metabolismo está regulado de manera muy sofisticada, y muchas de sus agrupaciones génicas no se expresan en condiciones estándar de laboratorio por diversas causas, como pueden ser que precisen de condiciones de cultivo muy específicas, la existencia de un represor ejerciendo su efecto en determinadas vías metabólicas, o incluso la necesidad de un estímulo adecuado (Liu *et al*., 2021).

En base al objetivo de activación de agrupaciones génicas silenciadas, motivado por la necesidad de encontrar nuevos compuestos bioactivos, en este trabajo, se aplican diferentes estrategias basadas en manipulación genética e interacción biológica. Las técnicas de manipulación genética están centradas en expresión heteróloga de reguladores globales y genes *housekeeping* conservados, así como en modificaciones mediante ingeniería ribosómica. Por otro lado, la estrategia de interacción biológica está basada en cocultivos con interacción mediada exclusivamente por compuestos volátiles, discerniendo así de los efectos ocasionados por la interacción mediada por la presencia de compuestos difusibles, lo cual constituye un enfoque mucho más novedoso. Todos estos experimentos se han realizado desde una aproximación OSMAC (empleando diferentes medios de cultivo) y ensayando una colección de actinomicetos aislados de un

ambiente poco explorado como es el tegumento de las hormigas cortadoras de hojas de la tribu *Attini* originarias de Perú. Esto implica un enfoque desde múltiples ángulos y combinando diferentes herramientas para tratar de aumentar la tasa de éxito en cuanto a la activación de rutas metabólicas se refiere. Además, estas son estrategias no dirigidas, es decir, de entre las herramientas presentadas en el apartado 2.7.1 de la introducción para la activación de agrupaciones génicas silenciadas, la totalidad de las aplicadas en este trabajo están enfocadas a estimular la activación de vías en general, sin centrar objetivos en una agrupación silenciada en concreto. A pesar de no estudiar un clúster en concreto, las herramientas bioinformáticas y en especial el uso del *software* AntiSMASH (Blin *et al*., 2023) han sido un factor clave en la identificación de los compuestos producidos por cada una de las cepas.

1-Co-expresión de reguladores globales y genes conservados

Se realizaron dos construcciones en el plásmido de expresión pSETxk usando una batería de cinco reguladores globales y cinco genes *housekeeping* con el objetivo de incrementar la producción de metabolitos, así como explorar la posibilidad de activar rutas metabólicas silenciadas. Estos 10 genes en total fueron amplificados a partir de ADN genómico de *S. coelicolor* empleando parejas de cebadores específicos con sitios de corte de enzimas de restricción únicos que permitieron su posterior clonaje secuencial. Así, los plásmidos pSETxkDCABA (conteniendo los genes que codifican para los reguladores pleiotrópicos *DraR, Crp, AbrC3, BldD y AfsR*) y pSETxkBMRRH (con los genes *housekeeping BldA*, *MetK, RpsL, RpoB, HrdB*) contenían una combinación de genes cuidadosamente seleccionados en base a los efectos reportados en la bibliografía sobre el metabolismo, ya que son genes conocidos por aumentar o activar la producción de ciertos compuestos en diversos estreptomicetos. Estas construcciones se diseñaron con la particularidad de la expresión heteróloga en bloque de varios genes a la vez, con el objetivo claro de activar o aumentar la expresión de vías metabólicas. En este ensayo no se busca determinar el gen o grupo de genes que provocan un efecto en concreto. La ingeniería de reguladores ha mostrado ser una herramienta eficiente en cuanto a la modificación del metabolismo para activar rutas silenciadas de metabolitos secundarios (Hao *et al*., 2024; Kang & Kim, 2021; Malla *et al*., 2010; Pei *et al*., 2024). Análogamente, mutaciones en genes conservados también han mostrado originar cambios favorables en

el metabolismo de *Streptomyces* (Gomez-Escribano & Bibb, 2012; Shaikh *et al*., 2021; Sun *et al*., 2017; Wang *et al*., 2013). La manipulación genética simultánea de varios de estos genes, además, está descrita como una estrategia que desencadena respuestas únicas sinérgicas diferentes a los efectos de la manipulación de los genes por separado, ya que estos interactúan entre sí (Mingyar *et al*., 2021; Sun *et al*., 2018).

El cultivo de los recombinantes generados se llevó a cabo en diferentes medios, variando su composición y la fuente de carbono y nitrógeno, debido a que esto puede ser determinante en la producción de compuestos. Se tomaron muestras de los caldos de cultivo a diferentes tiempos, así como se extrajeron los metabolitos de estos con diferentes solventes orgánicos, lo que también constituye un factor clave en la detección de nuevos compuestos, dado que así se diversifican las condiciones de análisis (Saw *et al*., 2021).

Como consecuencia de la expresión heteróloga de estos genes en las cepas de la colección CS se observaron diferentes respuestas metabólicas. Por un lado, se observó un aumento de producción de metabolitos con respecto a la cepa silvestre (datos no publicados) o al control conteniendo el plásmido vacío, como ocurre con las colismicinas y granaticinas en la cepa *Streptomyces* CS014 (Figura 3, Capítulo I). Este tipo de resultado es el cambio más frecuente que se ha observado en este ensayo. En consecuencia, en algunos casos se detectaron cambios en la bioactividad de las muestras, como se observa en los recombinantes de la cepa *Streptomyces* CS149 frente al control (Figura 7, Capítulo I). Un compuesto candidato como responsable de esta actividad es la colismicina, cuya producción es mayor en los recombinantes que en la cepa control. Sin embargo, estimamos que la actividad observada podría ser debida a otro compuesto no detectado por el análisis cromatográfico llevado a cabo, dado que no se detectaron diferencias de bioctividad frente a *E. coli* (se ha descrito que los compuestos de la familia bipiridil presentan actividad frente a Gram negativos) (García *et al*., 2013; Buckingham *et al*., 2010). Así mismo, también se detectó la síntesis de nuevos productos naturales que posteriormente fueron identificados por dereplicación, como es el caso de los compuestos tipo alteramida producidos por la cepa *Streptomyces* CS090a pSETxkBMRRH y las cromomicinas por *Streptomyces* CS065a pSETxkDCABA (Figura 8, Capítulo I). Como consecuencia de la activación de estos últimos compuestos, se observaron cambios en bioactividad frente a *M. luteus*. Desafortunadamente, todos los compuestos analizados resultaron ser metabolitos no solo ya descritos, sino que son producidos por la cepa control en otros medios de cultivo. No obstante, estos datos nos permiten apreciar cómo

la metodología seguida es efectiva para activar agrupaciones génicas silenciadas. También se observó el efecto opuesto en un caso aislado: la inhibición de la producción de un compuesto producido por la cepa control como consecuencia de la introducción del plásmido pSETxkDCABA en la cepa *Streptomyces* CS065a cultivada en SM10, afectando a la producción de cromomicinas (Figura 9, Capítulo I). Al igual que para los resultados de sobreproducción/activación, no se puede responsabilizar a ningún gen o grupo de genes de dicha actividad, aunque, dado que este cambio ocurre solo en uno de los recombinantes y en un medio en concreto, se puede deducir que existe un efecto represor combinado o no de los reguladores que afecta a la ruta de biosíntesis de las cromomicinas de manera muy específica. Por último, se detectó que la simple inserción del vector vacío también provocaba algún cambio metabólico, tanto con efectos positivos como negativos, dado que la introducción de genes que codifican la resistencia a antibióticos puede variar la producción del metabolismo secundario (Gehrke *et al*., 2019).

En definitiva, observamos todo tipo de respuestas metabólicas como consecuencia de la expresión heteróloga descrita en el Capítulo I, lo que además implica cambios en bioactividad, así como en la coloración de los cultivos (como ocurre en *Streptomyces* CS065a pSETxkDCABA en SM10). Solo existe un único caso de inhibición, aunque sí se ha observado en otros compuestos una disminución de la producción. En cualquier caso, en general, se observan muchos más resultados positivos (activación/sobreproducción), que negativos, cumpliendo así los objetivos iniciales del ensayo. Estos efectos observados *in vivo* muestran que los mismos reguladores tienen un efecto diferente en cada cepa y agrupación y, por lo tanto, no se pueden vincular efectos observados a uno o varios de los reguladores introducidos, ya que el entramado metabólico y las cascadas reguladoras que tienen lugar en cada cepa son complejas y permanecen siendo una incógnita.

Observando los resultados en particular, los efectos provocados son totalmente dependientes de cepa y medio, aunque también se puede observar como el cultivo de ciertas cepas en diferentes medios, dan lugar a respuestas metabólicas similares. Es el caso, por ejemplo, de la cepa *Streptomyces* CS057 pSETxkDCABA y pSETxkBMMRH: el cultivo de ambos recombinantes en los medios R5A, SM10 y SM17 resulta en la sobreproducción de skillamicinas, cicloheximida y actifenol. Aunque cada medio tiene una composición distinta, algunos comparten determinados sustratos, si bien en distintas proporciones (por ejemplo, R5A y SM17 contienen 10 g/L y 2 g/L de glucosa

respectivamente; SM10 contiene 23 g/L de glicerol, mientras que SM17 contiene 40 g/L). Dada la complejidad de la composición de los medios y la utilización de más de una fuente de carbono, no podemos extraer conclusiones basadas su composición. Sin embargo, merece ser mencionado esta repetición en los cambios metabólicos observados. También es importante señalar que los perfiles metabólicos de las cepas cultivadas en el mismo medio en su versión líquida y en su versión sólida no son iguales. Estos resultados nos sugieren la importancia de los procesos de *screening* o cribado en combinación con otras estrategias para desvelar la producción de nuevos compuestos, dado que las técnicas de manipulación genética pueden no ser suficientes a falta de unos requerimientos nutricionales mínimos.

De manera similar, se analizaron las respuestas metabólicas diferenciales al introducir estos plásmidos usando como cepas receptoras mutantes resistentes a estreptomicina (mutaciones en el gen *rpsL*) y rifampicina (mutaciones en el gen *rpoB*), obtenidos por ingeniería ribosómica, ya que es conocido que ambas mutaciones inducen la producción de metabolitos secundarios (Figura 10, Capítulo I) (Shima *et al*., 1996; Zhu *et al*., 2019). Este es un método no dirigido clásico, pero que en combinación con la expresión heteróloga de los genes reguladores y conservados en grupo podría ofrecer resultados de interés. Así, en los mutantes resistentes a rifampicina se observó un aumento de la producción de compuestos que fue mejorado con la introducción de los plásmidos, siendo estos resultados a su vez dependientes de cepa y medio de cultivo. Aunque la producción de compuestos resultó aumentada también en los mutantes resistentes a estreptomicina, esta producción no mejoró al introducirse ninguna de las dos construcciones. Esto pueden deberse a haberse alcanzado ya con la mutación inicial el límite máximo de producción.

Asimismo, usando la herramienta AntiSMASH se trató de determinar si existe relación entre la variación de la producción de un compuesto y la presencia en su agrupación de biosíntesis de sitios de unión para alguno de los reguladores empleados en este trabajo. Para la batería completa de sitios de unión detectados, en la Tabla 4 del Capítulo I se recogen aquellas agrupaciones modificadas con sitios de unión a estos reguladores, aunque, como se ha comentado previamente, no podemos asignar a ningún regulador como responsable de un cambio de producción en concreto.

En conclusión, presentamos estos dos plásmidos como una herramienta fácil de manipulación genética para realizar *screenings,* que favorece el aumento de producción, así como la activación de rutas silenciadas. En virtud de la necesidad de búsqueda de compuestos, que motiva el interés por la identificación de novedosas estructuras, el obtener una herramienta que promueva el aumento de producción de compuestos es un resultado esperanzador para facilitar la producción y análisis de nuevos compuestos sintetizados por estreptomicetos. Ciertos metabolitos secundarios son producidos en muy bajas concentraciones, lo que implica que no se pueden detectar por métodos de cromatografía líquida, y, por tanto, estudiar en condiciones adecuadas. Además, aunque un compuesto presente propiedades bioactivas de interés, debe ser obtenido en unas concentraciones mínimas para que su producción sea atractiva escala industrial y con ello su posterior empleo.

El estudio de nuevos compuestos bioactivos sobre cepas aisladas de entornos poco habituales, como este caso, suele ser una estrategia común en la actualidad. A partir de cepas de la colección CS, y en el contexto de compuestos difusibles, en nuestro grupo de investigación se han aislado varios productos naturales nuevos y se han obtenido derivados mejorados por biosíntesis combinatoria. Es el caso de las colibrimicinas producidas por *Streptomyce*s CS147 (Prado-Alonso *et al*., 2022) o las sipanmicinas producidas por *Streptomyces* CS149 (Malmierca *et al*., 2018; Malmierca *et al*., 2020). Sin embargo, es indudable que los *Streptomyces* spp. en estudio producen gran cantidad de compuestos ya conocidos, lo cual es esperable, dado lo ampliamente estudiada que está la producción de compuestos naturales por bacterias de este género. Las técnicas de dereplicación han ayudado a identificar rápidamente estos compuestos, evitando centrar esfuerzos en metabolitos ya conocidos. No obstante, muchos otros aún no han sido identificados y, por lo tanto, no están incluidos en bases de datos. Esto es un factor que limita mucho el proceso de investigación en esta línea. Lo mencionado anteriormente se observa con claridad en los resultados obtenidos de las diferentes cepas empleadas en nuestro estudio de co-expresión, donde muchos de los metabolitos no han sido identificados, aunque se hayan observado diferencias entre los cultivos en comparación con las cepas control.

Los compuestos que el análisis por dereplicación no ha logrado determinar siguen siendo una incógnita. Existen varios motivos por los que el análisis con la metodología empleada no ha podido identificar ciertos compuestos: pueden ser totalmente nuevos,

pero también pueden ser que no estén en la base de datos empleada, en las muestras a analizar puede encontrarse en bajas cantidades, o co-eluir con otros compuestos de manera que producen mezclas complejas difíciles de determinar. Es por esto, que, como proyecciones futuras de este trabajo, se debe intentar caracterizar aquellos compuestos no identificados. Para estos compuestos es necesario aplicar un cribado que determine las condiciones óptimas para su producción y posterior aislamiento conducente a su elucidación estructural. En cualquier caso, la estrategia planteada, ha demostrado ser eficiente para lograr incrementos de producción, lo cual es interesante desde un enfoque industrial, así como para la activación de rutas metabólicas, cumpliendo los objetivos iniciales del ensayo.

2-VOCs: herramienta como agentes inductores del metabolismo

Dentro de las estrategias de búsqueda de nuevos productos naturales, se encuentran los métodos de interacción biológica basados en cultivos combinados, en los que se aprovecha la comunicación entre microorganismos como herramienta para activar vías del metabolismo silenciadas (Onaka, 2017). A través de esta estrategia, se intenta imitar las condiciones ambientales de los microorganismos estudiados, ya que estos coexisten formando parte de comunidades complejas en las que intervienen multitud de variables.

En los últimos años, el estudio de los compuestos volátiles ha alcanzado un singular interés, dado que son moléculas que no han sido tan profundamente estudiadas como los compuestos difusibles por las dificultades de manipulación asociadas a las mismas, y la falta de métodos de análisis estandarizados. Sin embargo, se ha mostrado como estos metabolitos despliegan gran cantidad de propiedades bioactivas y tienen enormes implicaciones en comunicación celular, mecanismo que ha sido ampliamente descrito entre los diferentes reinos de los seres vivos (Abbas *et al*., 2022; Bouwmeester *et al*., 2019).

Diversos estudios han expuesto como, a partir de los compuestos volátiles producidos por un microorganismo, se han generado respuestas en otros como pueden ser estimular o inhibir la producción de ciertos metabolitos, el crecimiento, la formación de

esporas, inducir resistencia, modificar la motilidad, alterar la virulencia, etc. (Schulz-Bohm *et al*., 2017).

Debido a estos precedentes, se planteó un ensayo basado en la interacción de diversas cepas de *Streptomyces* de la colección CS entre sí, centrando esta comunicación solo en base a compuestos volátiles lo que permitiría diferenciar los efectos observados de aquellos que puedan estar provocados por compuestos difusibles. Para ello, se emplearon las cámaras de gases representadas en la Figura 1 del Capítulo II, que permiten el intercambio de compuestos gaseosos entre ambos microorganismos sin existir contacto físico ente ellos ni entre los compuestos difusibles que estos emiten al medio de cultivo. Esto pone en relieve una importante propiedad de los compuestos volátiles: son mensajeros que pueden actuar a distancia y ejercer su efecto en zonas más alejadas, incluso a las que pueden llegar por difusión aquellos compuestos no volátiles.

Los resultados obtenidos mostraron como diversidad de cepas aumentaron la producción de compuestos al ser expuestas a los agentes volátiles de otras cepas; tal es el caso de la granaticina en la cepa CS014 (en cocultivo con CS057, CS081a, CS90a, CS131 y CS149), la cicloheximida y actifenol en la CS057 (cocultivo con CS081a), o de un compuesto (o varios compuestos) no identificado en la CS207 (cocultivo con CS014, CS057, CS081a y CS227). También se observó la activación de rutas del metabolismo que se encontraban silenciadas: la colismicina en la cepa CS014 (en cocultivo con CS057, CS081a, CS90a, CS131 y CS149), las cosmomicina en la CS081a (cocultivo con CS014), las alteramidas y maltofilinas en la CS090a (cocultivo con CS057 y con CS081a), y las rumicinas 1 y 2 en la CS149 (cocultivo con CS131 y CS081a). Este último es el resultado más interesante obtenido, ya que los compuestos activados en las otras cepas son producidos en otras condiciones de cultivo (por ejemplo, en las expuestas en el Capítulo I). Sin embargo, era totalmente desconocido que la cepa CS149 fuera productora de rumicinas 1 y 2, y la agrupación responsable de su biosíntesis tampoco estaba descrita. En la mayoría de los casos (con la excepción de los compuestos no identificados producidos por la cepa CS207), los metabolitos activados por esta estrategia resultaron ser compuestos ya descritos, una vez más manifestando la problemática en la investigación de productos naturales que supone el continuo redescubrimiento de compuestos ya caracterizados.

Por otro lado, se evaluaron cambios en la morfología y crecimiento de las colonias a simple vista. El cultivo de las cepas en una extensión inferior a la totalidad de la placa permitió obtener información más clara sobre la morfología de las colonias de las diferentes cepas. No se observaron diferencias en la capacidad exploratoria, aunque sí se determinaron cambios en el momento de esporulación (cocultivos en comparación con los monocultivos). Existen varios estudios en los que se describen cambios en la capacidad de esporulación por efecto de los compuestos volátiles, incluso llegando a inhibirla. Ya en los años 60, Garrett y Robinson señalaron al ácido nonanoico como inhibidor de la formación de esporas (Gannett & Robinson, 1969). Así mismo, se observan notorias diferencias en el crecimiento en el caso de la cepa *Streptomyces* CS194 (Figura 2, Capítulo II), incapaz de crecer en SFM en monocultivo. Sin embargo, en cocultivo junto a otras CS, la cepa CS194 logró alcanzar diferentes niveles de crecimiento y desarrollo. Además, el cultivo junto a las cepas CS014, CS057, CS081a y CS227 estuvo acompañado de la producción de piperazinediones con actividad frente a *M. luteus*. Dada la ausencia de crecimiento en la muestra control, no podemos determinar esta actividad se debe a los compuestos volátiles que promueven el crecimiento, o también a aquellos que ejercen su efecto sobre el metabolismo.

En general, los resultados dependen de la cepa ensayada y la cepa vecina presente en el cocultivo, aunque el medio de cultivo puede ser un factor crucial, ya que el medio YMA parece inducir la producción de más volátiles. Es por esto por lo que se observa una clara diferencia de resultados de activación o aumento de la producción en los cultivos en YMA en comparación con los cultivos en los otros medios ensayados. En el caso del cultivo en el medio R5A, no se apreciaron diferencias y solo la CS149 y CS194 mostraron diferencias en SFM. Esto puede deberse a que en el medio YMA se favorezca la producción de compuestos difusibles mediante la activación ocasionada por estímulos volátiles de la cepa vecina; no obstante, los indicios apuntan a que también en el medio YMA se generan más volátiles inductores que en el resto de los medios ensayados. Para esclarecer este punto, se realizaron cultivos duales en diferentes medios de la cepa "emisora de volátiles" y de la "receptora de volátiles e inductora de nueva actividad". Aunque existía la posibilidad de que sean necesarias ambas condiciones (que se precise del cultivo en YMA de ambas cepas), se confirmó que el factor clave era la producción de determinados volátiles en medio YMA.

Otro resultado interesante es el obtenido por el cultivo múltiple de las cepas en grupos de cuatro, observando resultados no obtenidos por el cultivo dos a dos de estas mismas cepas, como por ejemplo sucede en el caso de la cepa *Streptomyces* CS065a, donde se activan la producción de alteramidas y cromomicinas al ser cocultivada con otras cepas adicionales. Esto lleva a pensar en la necesidad de la acción combinada de más de un compuesto volátil para la activación de rutas o aumento de producción, o de mayor cantidad de un mismo compuesto volátil ejerciendo su efecto. Este ensayo nos sugiere que, en comunidades bacterianas complejas, la emisión de estos mensajeros químicos de acción rápida permitiría un intercambio de información incluso a cierta distancia que puede provocar diferentes tipos de respuestas en los organismos receptores, provocando incluso cambios que no se consiguen replicar en el laboratorio en monocultivo, ni en cultivos duales tal como hemos podido observar.

En una visión general, examinando los resultados obtenidos en este ensayo y en los estudios de expresión heteróloga comentados en la sección anterior, observamos cómo hay ciertos genes implicados en la biosíntesis de metabolitos secundarios que activan su expresión con diferentes estrategias. Mientras que la activación de la ruta de síntesis de las rumicinas en *Streptomyces* CS149 solo lo hemos logrado mediante el empleo de compuestos volátiles de otras cepas, la activación de las alteramidas en *Streptomyces* CS090a ha ocurrido por acción de los volátiles de *Streptomyces* de CS057 y CS081a, así como por la introducción del plásmido pSETxkBMRRH y cultivo en R5A. Esto pone en relieve algo ya previamente descrito, y es que ciertas agrupaciones parecen ser más fáciles de activar que otras (Li *et al*., 2018), tal vez por estar sometidas a un control regulatorio menos estricto.

Este ensayo muestra una realidad ya conocida, aunque poco trasladada a nivel experimental en el laboratorio. Los organismos conviven en sistemas complejos en los que intervienen multitud de factores bióticos y abióticos. Las técnicas clásicas de análisis en microbiología se han basado en cultivos puros, sin evaluar las interacciones bióticas. A pesar del éxito obtenido en la activación de BGCs con tal enfoque, no se debe ignorar que muchos de los metabolitos microbianos de interés se producen para una actividad específica, como por ejemplo puede ser la producción de antibióticos como arma mediadora en relaciones de competencia, o la producción de antioxidantes como mecanismo de defensa contra los rayos UV (Tan *et al*., 2019; Weiland-Bräuer, 2021). Aunque es imposible mimetizar las condiciones cambiantes del medio ambiente, merece

ser destacada esta complejidad del sistema y el entramado metabólico que se desarrolla en estos ecosistemas.

En el presente estudio se evalúan exclusivamente las interacciones dos a dos, así como en grupos de cuatro solo teniendo en cuenta los compuestos volátiles; no se han tenido en cuenta otras interacciones reales que sí ocurren en el ambiente como son las interacciones físicas entre organismos o las interacciones por compuestos difusibles. En cualquier caso, el efecto de los compuestos volátiles de manera aislada es suficiente para generar cambios metabólicos de diferente tipo en otros organismos, lo que podría ser empleado como estrategia en los programas de descubrimiento de nuevos productos naturales. Además, se identifican respuestas similares en cocultivo con diferentes microorganismos, lo que sugiere que diferentes cepas producen un mismo compuesto volátil o que una vía metabólica en particular responde de manera similar ant diferentes compuestos volátiles. De hecho, en un ensayo realizado por nuestro grupo no incluido en esta tesis doctoral, en el que se replican estos ensayos cocultivado las cepas de la colección CS con actinobacterias no estreptomicetos, se repiten algunos resultados ya expuestos aquí, como es el caso de la activación de las rumicinas en el cocultivo de la cepa CS149 al cocultivarla en YMA frente a *Micromonospora melanospora* ATCC3104 (datos pendientes de publicación). Esto refleja que ambas actinobacterias producen algún compuesto volátil que desencadena de manera específica la misma respuesta. Se necesitan más estudios para poder esclarecer este punto, y poder identificar la molécula/s responsable de originar dichos cambios.

En resumen, el estudio de las interacciones de microorganismos mediadas por compuestos volátiles es una estrategia eficiente para explotar el potencial metabólico del género *Streptomyces*, ya que muchas de estas moléculas pueden actuar como inductores y desencadenar la activación de rutas metabólicas silenciadas. Hasta ahora se ha descrito una gran selección de respuestas metabólicas generadas por el efecto de los compuestos volátiles. Sin embargo, los mecanismos de regulación subyacente de esta actividad siguen siendo una incógnita. En la mayoría de los estudios se han definido las interacciones entre especies en condiciones específicas y los consecuentes efectos de las mismas, pero se desconoce que compuesto o compuestos son los responsables de originar estas respuestas, por lo que sería de gran interés descubrir la naturaleza de estas moléculas señalizadoras y los mecanismos de reconocimiento de estas señales para tener una visión global y completa de los procesos de interacción microbiana. Además, el conocimiento de esta red

de comunicaciones no solo es útil aplicado al mundo microbiano, sino que también puede ser aplicado a las relaciones interespecíficas, ya que los microorganismos interaccionan con otros grupos de organismos tales como plantas, hongos, parásitos, animales etc., incluso un mismo compuesto puede ser producido por varios de estos grupos mencionados.

3-Propiedades antifúngicas de los volátiles de *Streptomyces* y su papel en los nidos de hormigas cortadoras de hojas

Como se ha comentado anteriormente, el estudio de los compuestos volátiles ha despertado un gran interés debido a su implicación en mediación de la comunicación entre organismos. El ecosistema del que se han aislado los *Streptomyces* empleados en este trabajo es muy particular. Por un lado, se encuentran las hormigas cortadoras de hojas, que conviven en un sistema muy jerárquico y dependen del hongo *L. gongylophorus* para su alimentación. Por otro lado, este último no es el único representante fúngico en dicho ambiente, ya que le acompaña el patógeno *E. weberi* (entre otros, aunque es el género más destacado y específico) atacando el equilibrio del sistema. Es aquí donde los actinomicetos, entre ellos los estreptomicetos, juegan un papel fundamental en la defensa del sistema al completo, donde intervienen múltiples organismos y se establecen gran variedad de interacciones de distinto tipo (Figura 14). Además, en ambientes confinados como los nidos de estas hormigas, con una arquitectura compleja basada en múltiples cámaras de aire, cabe pensar que los compuestos volátiles tienen un enorme papel en comunicación celular.

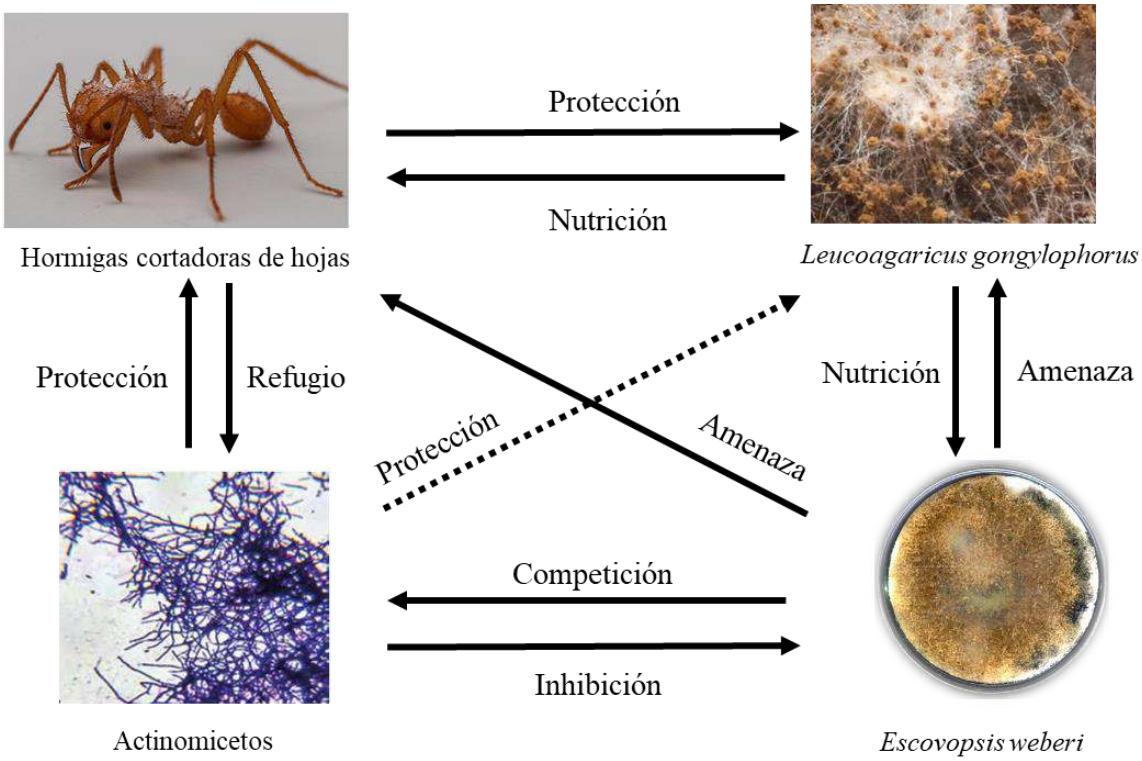

Figura 14: esquema de las interacciones que se establecen en los nidos de las hormigas cortadoras de hojas (Gutierrez-Espinoza & León-Quispe, 2018; Holmes *et al*., 2016; Pagnocca *et al*., 2012; Steffan *et al*., 2015).

En este entorno, la producción de antimicrobianos difusibles es muy importante. Sin embargo, estos compuestos tienen un rango de acción mucho menor que los compuestos volátiles. En contraposición, estas últimas son moléculas de acción química rápida y de mayor alcance (pueden viajar más lejos que los compuestos difusibles) (Schmidt *et al*., 2015; Schulz-Bohm *et al*., 2017). Dado que en los nidos de hormigas los diferentes tipos de microorganismos no tienen por qué estar en contacto físico entre sí, puede que los compuestos difusibles no tengan suficiente rango de acción para ejercer su efecto. Esto es especialmente importante dado que *E. weberi* es un hongo de crecimiento muy rápido por lo que la emisión de semioquímicos (sustancias químicas producidas por organismos que provocan respuestas en individuos de la misma o de otra especie) (De Mana *et al*., 2016; Rico-Martínez *et al*., 2022) es fundamental para controlar y modular el comportamiento de los individuos vecinos, determinando la supervivencia de determinadas especies o géneros en este ambiente específico, así como su convivencia con otros organismos (Jones *et al*., 2019; Tyc *et al*., 2015).

En un ensayo inicial, se evaluó el potencial antifúngico total que presentan los microorganismos de la colección CS frente a *E. weberi* en un cocultivo en la misma placa, en los medios de cultivo R5A, SFM y PDA. En los casos en los que el desarrollo del hongo no es total, este efecto puede ser debido a la acción combinada de los compuestos difusibles y de los compuestos volátiles. Para determinar el efecto antifúngico exclusivo generado por los volátiles, diferentes cepas de *Streptomyces* de la colección CS se cocultivaron en cámaras de intercambio de volátiles frente a *Escovopsis*. De este modo, se determinó que en medio SFM las cepas *Streptomyces* CS014, CS057, CS131, CS147, CS159, CS207, y CS227 inhiben o retrasan el crecimiento del mismo mediante la acción exclusiva de estos compuestos. Para el estudio de la naturaleza de las moléculas responsables de este efecto se añadió carbón activo a las cámaras de gases. Posteriormente, los metabolitos capturados se extrajeron con acetato de etilo y se analizaron por GC-MS (cromatografía de gases/espectrometría de masas).

Como muestras control del ensayo, se establecieron el cultivo del hongo en monocultivo, *Streptomyces* en monocultivo en R5A y en SFM, y *Streptomyces-E. weberi* en cocultivo en R5A. Los volátiles producidos en SFM por ambos microorganismos en monocultivo permiten diferenciar si producen compuestos nuevos cuando se produce el cocultivo. Los tres ensayos en R5A (ambos microorganismos en monocultivo y el cocultivo) sirven para identificar el/los potencial/es compuestos antifúngicos: aquellos compuestos que estén producidos en SFM que también sean producidos en R5A deducimos que no son los responsables de la actividad antifúngica, ya que en R5A no hay inhibición micótica.

Los resultados obtenidos muestran como las diferentes cepas producen variedad de metabolitos con propiedades antifúngicas previamente descritas. Entre ellos se encuentran productos del metabolismo primario y secundario, así como compuestos orgánicos e inorgánicos. El análisis comparativo de los compuestos producidos por cada cepa de *Streptomyces* en monocultivo y aquella expuesta a *E. weberi* revela que, ante la presencia del hongo patógeno, los actinomicetos comienzan a producir nuevos compuestos en respuesta. La combinación de la producción de estos nuevos compuestos, junto con otros volátiles que intrínsecamente son producidos por *Streptomyces*, generan un ambiente inhóspito para el crecimiento y desarrollo del patógeno del jardín fúngico. Por tanto, ciertos individuos de la colección CS inhiben o retrasan el crecimiento de *Escovopsis* mediante la acumulación de compuestos con propiedades antifúngicas.

Tradicionalmente, las estrategias de cribado en búsqueda de nuevos compuestos bioactivos, una vez identifican una actividad de interés, buscan la caracterización del compuesto responsable de dicha actividad. En este caso, planteamos que la inhibición fúngica es debida a la actividad aditiva o sinérgica de varios volátiles con potencial antimicrobiano, y no atribuimos esta actividad a ningún compuesto en particular. Existen compuestos como el cis-Muurola-4(15),5-dieno que forman parte de aceites esenciales con actividades antimicrobianas y que están presentes en diferentes muestras obtenidas tanto en medio SFM como en R5A. Esto implica que este tipo de compuestos no son los únicos responsables de la actividad antifúngica, pero no podemos negar que contribuyan a generar este ambiente tan poco propicio para el crecimiento del hongo. Por lo tanto, en medio R5A también se producen diversos compuestos con capacidad antifúngica, pero no se producen tantos compuestos con la actividad requerida o no se generan en suficiente cantidad para que su efecto sea apreciable. Además, se observa como este efecto es resultado de una actividad combinada de volátiles cuando se exhibe actividad frente a *Escovopsis* por parte del cocultivo de cepas de *Streptomyces* que no mostraron actividad inhibitoria en el ensayo inicial de manera individual (Figura 10, Capítulo III). Así, la acumulación de compuestos producidos por *Streptomyces* CS090a y CS081a limita el crecimiento del hongo, probablemente por la saturación del ambiente por compuestos antimicrobianos.

Por otro lado, está claro que los estreptomicetos elaboran respuestas ante la presencia del hongo y hemos evaluado el potencial antifúngico de las mismas. Este hecho no solo ha sido corroborado por el cambio manifiesto en la producción de volátiles, sino que, además, se han observado cambios en la producción de compuestos difusibles (Figura S3, Capítulo III, Material suplementario). En general, se determina que la presencia fúngica genera una respuesta que implica cambios metabólicos precisos. Una opción interesante como proyección futura sería obtener fracciones de la producción de compuestos para cada cepa y evaluar el potencial antifúngico de cada una de ellas por separado.

De forma adicional, cabe destacar que muchos de los metabolitos identificados en este ensayo están descritos como componentes de aceites esenciales de plantas. Este hecho es algo ampliamente conocido, ya que, por ejemplo, se sabe que hongos y bacterias son productores de compuestos flavonoides, metabolitos secundarios típicos de plantas

(Fanele & Ndlovu, 2023; H. R. Kang *et al*., 2016; Wang *et al*., 2020). Sin embargo, se desconoce si esas moléculas específicas exhiben una actividad en concreto.

Es importante resaltar que muchos de los compuestos volátiles producidos por las cepas seleccionadas para el análisis por GC-MS son comunes, así como las predicciones ofrecidas por AntiSMASH para la síntesis de terpenos. No todos los terpenos son volátiles, ni todos presentan actividad antifúngica, aunque sí se ha prestado especial atención a la producción de terpenos dado las grandes propiedades asociadas ellos. La coincidencia de metabolitos producidos entre las diferentes cepas es lógica, dado que pertenecen al mismo género y además son organismos aislados del mismo ambiente. También es importante tener en cuenta que los volátiles expuestos y analizados en este trabajo no son la totalidad de los compuestos que producen las cepas y, por tanto, los que ejercen el efecto sobre el hongo. Esto es debido a las limitaciones de la metodología llevada a cabo (método de absorción, extracción con acetato de etilo, columna empleada en el análisis por GC-MS, librería empleada). Un ejemplo de compuesto no identificado por esta metodología es el amonio, que hemos demostrado mediante otros ensayos complementarios que sí es producido por estas cepas y el cual ha sido previamente asociado como uno de los responsables de ejercer inhibición sobre el crecimiento de *E. weberi* (Dhodary & Spiteller, 2021).

Por otro lado, se determinó cómo estos volátiles antifúngicos no presentan efecto frente a otros hongos como son *P. cinnamomi* o *Sclerotinia* sp., lo que representa un resultado intrigante, pero que puede sugerir, considerando las implicaciones ecológicas de los metabolitos producidos por actinomicetos, cierta especificidad respaldada por componentes particulares para atacar al organismo problemático del ecosistema de donde estas cepas fueron aisladas y no a *L. gongylophorus*. No es posible asegurar una exclusividad del efecto antifúngico observado, pero al menos este no es efectivo sobre todo tipo de hongos. Dhodary y colaboradores (Dhodary & Spiteller, 2021) determinaron que este efecto podría ser resultado de la producción de amonio por las diferentes cepas, ya que *E. weberi* es altamente sensible a la basificación del entorno. Esta es la razón por la que planteamos determinar si las cepas de esta colección eran productoras de amonio. Observamos a través del ensayo directo con rojo de fenol, como efectivamente eran productoras de un metabolito que basificaba el ambiente. Posteriormente, se realizó un ensayo basado en la reacción de Berthelot, en el que se comprobó como realmente este compuesto era amonio (Gordon *et al*., 1978). Este experimento se basa en la adición de

hipoclorito y fenol a placas expuestas a los volátiles de las diferentes cepas, que en presencia de amonio reaccionan para dar lugar a la producción de indofenol, compuesto de coloración azul. Como ejemplo, en la Figura 15 se muestra el resultado de este experimento sobre la cepa *Streptomyces* CS131, una de las que ejerce un efecto inhibitorio más notorio sobre *Escovopsis*.

Figura 15: reacción de Berthelot sobre el medio de cultivo de una placa expuesta a los volátiles de la cepa *Streptomyces* CS131 (derecha) y una placa sin exposición a volátiles (izquierda).

Tal como ha sido expuesto en la Figura 11 del Capítulo III, la cepa CS081a es productora de amonio, pero esta no es una de las cepas que causa retraso o inhibición del crecimiento de *Escovopsis*. En contraposición, la cepa CS227 no es productora de amonio, pero sí exhibe actividad antifúngica. Debido a esto, deducimos que el amonio es una molécula más que contribuye a producir un ambiente poco adecuado para el crecimiento del hongo, pero la inhibición no depende exclusivamente de este factor. Además, de manera complementaria, se realizó la eliminación del gen *gcvP* para interrumpir la vía de producción de amonio en estas cepas y determinar si esta modificación revierte el efecto inhibitorio micótico (la interrupción de la ruta se comprobó por la reacción de Berthelot). Acorde la hipótesis inicial planteada, el efecto inhibitorio se mantuvo, lo cual la confirma que el amonio no es la única molécula responsable de la actividad antifúngica (datos pendientes de publicación). En la Figura 16 se observa este ensayo sobre la cepa *Streptomyces* CS131, que es productora de amonio.

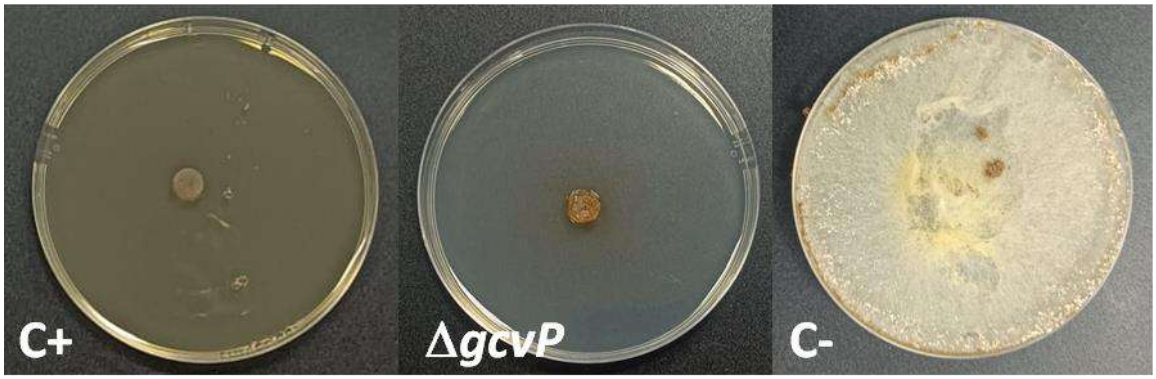

Figura 16: crecimiento de *E. weberi* al ser cocultivado frente a *Streptomyces* CS131 (C+), *Streptomyces* CS131 Δ*gcvP,* y en monocultivo (C-). Se observa como la inhibición persiste a pesar de la interrupción de la ruta de escisión de la glicina.

Como visión general de este trabajo, entre todas las estrategias empleadas para tratar de activar rutas metabólicas silenciadas, el estudio de los compuestos volátiles se ha convertido en un área de interés, y no solo por las potenciales propiedades biológicas de los mismos, sino también su implicación en comunicación con otros organismos. Múltiples campos de la industria se han aprovechado de las importantes propiedades asociadas a los agentes volátiles: en agricultura como promotores del crecimiento, en alimentación como indicador de calidad y en medicina para la detección de enfermedades.

En los nidos de hormigas cortadoras de hojas, se dan multitud de interacciones tanto inter- como intraespecíficas y de diferente tipo (competencia, mutualismo, etc.). En este ecosistema la función de los compuestos volátiles es crucial para mantener controlado el crecimiento de *E. weberi*, pudiendo actuar a más allá del rango de acción de los compuestos difusibles y manteniendo así el equilibrio del sistema. El análisis de la producción de estos compuestos por parte de los estreptomicetos de la colección CS reveló la producción de gran cantidad de volátiles; la acumulación del efecto combinado de todos ellos reflejó en ciertos casos la inhibición o retraso del hongo patógeno del jardín fúngico. Esta actividad biológica convierte al volatiloma de estos estreptomicetos en elemento esencial para el correcto mantenimiento del medio ambiente, y su ausencia podría provocar un desequilibrio con el consiguiente efecto dominó sobre otros miembros de ese nicho ecológico o cadena trófica.

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V. Conclusions/ Conclusiones

1-The use of heterologous expression of global regulators and housekeeping genes allows an increase in the production of compounds as well as the activation of silenced metabolic pathways, which is a strategy of interest in the process of discovery of new natural products.

2-Inter- and intra-specific signaling plays a key role in cellular development and metabolism regulation. Interactions mediated by volatiles are presented as a strategy to exploit the metabolic potential of microorganisms and activate silent gene clusters given its implications in cellular communication.

3-There is a complex network of interactions mediated by Volatile Organic Compounds (VOCs) that is essential for the maintenance of ecosystems. The chemical properties of these compounds make them the perfect form of communication in an environment full of air spaces like the ground.

4-Inhibition of *Escovopsis weberi* by *Streptomyces* sp. could be due to the accumulation of compounds with antifungal properties.

5-The presence of *E. weberi* generates a response in the metabolism of *Streptomyces*, leading to the production of new volatile compounds.

6-The use of VOCs chambers has been successfully applied to study the effect of volatiles, differentiating their effects from those produced by diffusible compounds, becoming a strategy for growth promotion, metabolites overproduction, and drug discovery.

1-El empleo de la expresión heteróloga de reguladores globales y genes *housekeeping* permite un aumento en la producción de compuestos así como la activación de vías metabólicas silenciadas, siendo una estrategia de interés en el proceso de descubrimiento de nuevos productos naturales.

2-La señalización inter- e -intraespecífica juega un papel clave en el desarrollo celular y la regulación del metabolismo. Las interacciones mediadas por volátiles se presentan como una estrategia para explotar el potencial metabólico de los microorganismos y activar agrupaciones de genes silenciosas dadas sus implicaciones en la comunicación celular.

3-Existe una red compleja de interacciones mediadas por Compuestos Volátiles Orgnánicos (VOCs) que es esencial para el mantenimiento de los ecosistemas. Las propiedades químicas de estos compuestos los convierten en la forma perfecta de comunicación en un entorno lleno de espacios aéreos como el suelo.

4-La inhibición de *Escovopsis weberi* por *Streptomyces* sp. podría deberse a la acumulación de compuestos con propiedades antifúngicas.

5-La presencia de *E. weberi* genera una respuesta en el metabolismo de *Streptomyces*, dando lugar a la producción de nuevos compuestos volátiles.

6-El uso de cámaras de VOCs se ha aplicado con éxito para estudiar el efecto de los volátiles, diferenciando sus efectos de los producidos por compuestos difusibles, convirtiéndose en una estrategia para la promoción del crecimiento, la sobreproducción de metabolitos y el descubrimiento de fármacos.

VI. Informe publicaciones

Base de datos de indexación: Science Citation Index (JCR-SCIE)

Capítulo I

Cuervo, L.; Malmierca, M.G.; García-Salcedo, R.; Méndez, C.; Salas, J.A.; Olano, C.; Ceniceros, A. Co-Expression of Transcriptional Regulators and Housekeeping Genes in *Streptomyces* spp.: A Strategy to Optimize Metabolite Production. *Microorganisms* 2023, *11*(6), 1585. https://doi.org/10.3390/microorganisms11061585. PMID: 37375086; PMCID: PMC10301460.

Año: 2022

Editorial: MDPI

Factor de impacto: 4.5

Categoría: Microbiology

Posición que ocupa la revista en la categoría: 47/135

Cuartil: Q2

Capítulo II

Cuervo, L.; Méndez, C.; Salas, J.A.; Olano, C.; Malmierca, M.G. Volatile Compounds in Actinomycete Communities: A New Tool for Biosynthetic Gene Cluster Activation, Cooperative Growth Promotion, and Drug Discovery. *Cells* 2022, 11(21), 3510. doi: 10.3390/cells11213510. PMID: 36359906; PMCID: PMC9655753.

Año: 2022

Editorial: MDPI

Factor de impacto: 6.0

Categoría: Cell Biology

Posición que ocupa la revista en la categoría: 60/191

Cuartil: Q2

Capítulo III

Cuervo, L.; Álvarez-García, S.; Salas, J.A.; Méndez, C.; Olano, C.; Malmierca, M.G. The Volatile Organic Compounds of *Streptomyces* spp.: An In-Depth Analysis of Their Antifungal Properties. *Microorganisms* 2023, 11(7):1820. doi: 10.3390/microorganisms11071820. PMID: 37512992; PMCID: PMC10384482.

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Posición que ocupa la revista en la categoría: 47/135

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