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光桿菌 *Photorhabdus akhurstii* 防治草莓炭疽病產生之有效物質特性分析

Characterization of active compounds against strawberry anthracnose produced by *Photorhabdus akhurstii*

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本論文係邱倢緗君（學號 R06B42004）在國立臺灣大學植物科學研究所完成之碩士學位論文，於民國 108 年 7 月 16 日承下列考試委員審查通過及口試及格，特此證明

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誌謝

轉瞬之間，我的研究所生涯即將劃下句點，要邁入下一個人生階段。這兩年的時間是我當學生身份學習最多的階段，不管是待人處事上，亦或是自己獨自一人在異鄉念書，都有莫大的收穫和感受，而我能得到這個學位及撰寫完成論文並完成實驗，實在有許多需要感謝的人事物！

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姊在 meeting 上常給予我實驗上的建議及提供我問題參考，使我思考許多自己未曾想過的層面。而在我口試前，很感謝星怡學姊和昆縉學長協助我修正報告的投影片，以及婉嘉不厭其煩地聽我練習，使我的報告更臻完善。還要感謝琇銀學姊和 Sandy 學姊平時幫我們訂購藥品及處理許多繁瑣的大小事，學姊總是很親切耐心地協助我們、謝謝雪花姊幫我們清理及準備許多實驗上會用到的器皿，使我們可以省下很多時間專心做實驗。謝謝微生物組的學長姊阿 Ben、姿妍、薏安和岱庚，給予過我實驗上的協助和材料上的支援。也謝謝在研究所期間同實驗室的好同學婉嘉，不管是課業或是生活上總很貼心地幫助了我許多事情，我們常常替彼此加油打氣及吃飯紓壓，實在很幸運在就讀研究所期間遇見婉嘉！

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我覺得我很幸運，在我就讀研究所期間，能受到這麼多人的協助和幫忙。也因著大家給予我的福氣使我順利的完成口試和論文撰寫。而我也想把我自己的收穫及喜悅化成祝福送給每一個在研究所期間幫助過我的人，祝福大家不管在研究的領域上或是業界的發展，也都能幸運、順心平安。

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摘要

草莓是一高產量且高經濟價值的作物，臺灣主要栽培草莓地區為苗栗縣大湖鎮，在栽培過程中如遇上高溫高濕度之環境會引發草莓炭疽病(Strawberry anthracnose)；主要是由 *Colletotrichum gloeosporioids* (Cg) 病原菌所造成，會造成草莓經濟及收成有極大的損失和影響。在過去的病害防治上，多使用化學農藥來進行防治，然而過度的使用會導致抗藥性產生、食安問題以及藥劑對農民健康的危害。因此期望生物農藥可以成為替代的方案。本研究首先在對峙平板上確立了液化澱粉芽孢桿菌(*Bacillus amyloliquefaciens*) ASB111 和光桿菌第一型(*Photorhabdus akhurstii* sp. nov. 0813-124 phase I) PL1 有顯著的抑制效力，且 PL1 對其它 *Colletotrichum* 屬的病原菌也具有防治效力。基於研究的潛力及 PL1 防治草莓炭疽病的機制尚未明確，PL1 成為我們主要進行研究的對象。在建立穩定的培養條件實驗中，得知 PL1 在經過 24 小時培養的缺鐵培養基(LP)中有最高的抑制效力，然而 PL1 在 PDB 液態培養基中未能被培養出。有趣的是，PDB agar (PDA) 在固態培養基的選擇試驗上顯示能使 PL1 產生有效物質去抑制 Cg。另外，在最佳的萃取條件實驗中，顯示以乙酸乙酯(EA)萃取挖下的 agar 菌盤能獲得較高效率的粗萃物。經由高效液向層析儀(HPLC)分離純化 PL1 乙酸乙酯粗萃物，再透過液相質譜儀與串聯式質譜儀獲得之 MS^1 與 MS^2 數據進行分析，整合了二次質譜碎片進行結構預測，得到主要的抗生物質為 glidobactin A, cepafungin I 和 glidobactin C，它們均屬於 syrbactins 家族的化合物。除此之外，根據一次質譜的層析圖，這類的有效物質會受到 agar 固態培養的方式被誘導產生，且 PDA 能誘導比 LPA 更多的有效物質。利用質譜儀所得之結果結合全基因組定序；在全基因組定序經由 antiSMASH 的分析結果，PL1 和 PL2 都有 22 個生合成基因簇，第 8 基因簇中有 26%的基因與 glidobactin 具有相似、第 15 基因簇中也有 15%的基因與 glidobactin 具有相似，我們猜測這可能與 glidobactin 的生合成路徑有關，然而這些生合成基因尚需進一步的實驗加以驗證。

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關鍵字：草莓炭疽病、共生細菌、光桿菌、生物防治、glidobactin、cepafungin、生合成路徑

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Abstract

Strawberries is an important crop and generates substantial economic values for farmers in Taiwan. *Colletotrichum gloeosporioides* (Cg) is one of the main pathogens in the cultivation of strawberry, which is also called strawberry anthracnose. This disease causes severe loss in strawberry production. Spraying the pesticides is a general practice to reduce the loss caused by anthracnose in the field. However, overuse of the chemical pesticides brings many problems such as the pollution in environments, emergence of drug-resistant strains, food safety risks and the health of the farmers. So, biological control is proposed as an alternative approach to replace chemical pesticides. In this study, the antagonistic assays showed that *Bacillus amyloliquefaciens* strain ASB111 and *Photorhabdus akhurstii* sp. nov. 0813-124 phase I (PL1) had anti-Cg ability. In addition, PL1 also had the ability against several other *Colletotrichum* spp. PL1 was chosen for further analyses to identify novel anti-Cg compounds and to elucidate the mechanisms for PL1 against Cg. PL1 grown in the iron limited medium (LP) at the first 24hr culture time had the highest inhibitory activity, and PL1 could not grow in potato dextrose broth (PDB). Interestingly, PL1 was induced to produce active compounds in PDB agar (PDA). Among different extraction methods, the cut-agar method and EA extraction had the best extraction efficiency. The active compounds of EA extracts were purified by high-performance-liquid-chromatography (HPLC) and identified by high resolution and high

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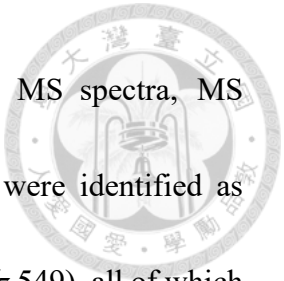

mass accuracy experiments (Mass spectrometry). By integrating MS spectra, MS fragments and structure prediction, the active compounds of PL1 were identified as glidobactin A (*m/z* 521), cepafungin I (*m/z* 535) and glidobactin C (*m/z* 549), all of which belong to the syrbactin family. Base on MS spectra, active compounds of syrbactin family could be highly extracted in the cut-agar extraction. The active compounds might be induced by the incubation on the PDA. In addition, PL phase II (PL2) did not have anti-Cg ability. According to the anti-SMASH analysis of whole genome sequencing, PL1 and PL2 had 22 biosynthetic gene clusters (BGCs). Among the clusters, region 8 showed 26% of genes were similar to glidobactin and region15 showed 15% of genes were similar to glidobactin. Thus, we hypothesize that the different antagonistic abilities of PL1 and PL2 might be caused by differential expression of genes in the syrbactin family biosynthetic pathway.

KETWORDS: Strawberry anthracnose, symbiotic bacteria, *Photorhabdus akhurstii*, biocontrol, glidobactin, cepafungin, biosynthetic pathway

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Introduction

Strawberry industry overview

Strawberry is a Rosaceae herbaceous plant and was classified to berry fruits. *Fragaria* × *ananassa* is the major cultivar that is a hybrid of two native species, *F. chiloensis* and *F. virginiana* (Hancock et al., 2008). Strawberry has abundant aroma and vitamin C. It also contains ellagic acid which is a natural phenolic antioxidant. According to Food and Agriculture Organization of the United Nations (FAO) data, worldwide strawberries cultivated area was around 390,000 hectares and the production was over 9.22 million tons in 2017 (FAO, 2017). China was the top producer which produced 3717283 tons strawberries followed by United States of America, Mexico, Spain and Jordan. Therefore, strawberries are high economic crops and high production crops.

In Taiwan, strawberries cultivated area was around 506 hectares and the production was 8 thousand tons (Council of Agriculture, Executive Yuan, 2018). Miaoli County ranks first in the cultivated area. Meanwhile, the strawberries industry brings rich tourism income to Miaoli each year. The most common cultivars of Taiwan is Taoyuan#1（桃園 1 號）which was introduced from Japan at 1985 and also called Fong-Hsiang（豐香）according to TARI（Taiwan Agricultural Research Institute). The Fong-Hsiang strawberries grows vigorously and the leaves are thick and green, so it has become a popular cultivar for strawberry growers.

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Strawberry growth conditions and diseases

Strawberry cultivars grown in different regions are adapted to the day length and temperatures. Among them, the cultivated temperatures have a huge effect on plant growth, anthocyanin content and fruit production (Zhang et al., 1997). The optimal temperature range for strawberries to grow and fructify is 18~27°C, however, many strawberry cultivars can adapt to the area which has low temperatures in winter at 5~17° C (TARI). The cultivation can be divided into two stages, the nursery stage and the field growth stage. In Taiwan, the strawberries enter the vegetative phase after April when the temperature gradually increases. At the same time, both old plants and new seedlings of strawberries will sprout the runners and then the farmers can take the runners to cultivate. On top of that, Taiwan has more advantages than other low-latitude subtropical regions for cultivating strawberries, because Taiwan has cool temperatures in winter, long daylight and low relative humidity in summer (李，2011). Therefore, strawberries are cultured from early May to middle September and planted in the field from the end of September to October in Taiwan (李和呂，2004).

In recent years, global warming causes climate change such as temperature change and unstable rainfall that cause damages to the crops. Besides, the occurrence of high temperature and heavy rains during the nursery stage may cause serious diseases, especially the strawberry anthracnose, in Taiwan.

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Strawberry anthracnose

Anthracnose is caused by *Colletotrichum* spp., which belongs to Fungi, Ascomycota, Sordariomycetes, Glomerellales, Glomerellaceae and *Colletotrichum* (Reblova et al., 2011). According to some studies, strawberry anthracnose could be caused by several species, such as *C. thebromicola*, *C. acutatum*, *C. gloeosporioides* and *C. dematium* (Howard et al., 1992). The strawberry anthracnose was first discovered in Miaoli County (李和呂，1994). The species in my thesis is *Colletotrichum gloeosporioides*, which is provided by Dr. Feng-Chia Hsieh. *Colletotrichum gloeosporioides* belongs to the hemibiotrophic pathogen. *C. gloeosporioides* cause lesions on strawberry roots, stems, flowers, fruits and leaves. The pathogen also causes post-harvest infection (Prusky and Plumbley, 1992). The optimal temperature for *C. gloeosporioides* growth is 25-28°C and pH 5.8-6.5 (Sharma and Kulshrestha, 2015). In the beginning, it colonizes with white mycelia, then changes to orange type conidia and completes its growth around 7 days on PDA 9 cm plates in lab studies (growth rate 1.2cm/day). The sources of Cg infection include transplant seedlings, the residues of plants, soils and intermediate host. The spores germinate on the surface of strawberry and produce appressoria that can produce hyphae to infect the host in the late infective process. And the lesion appears as small, dark spots on stolons and petioles (Sharma and Kulshrestha, 2015). During an epidemic of the disease, it even causes strawberry dead. Owing to strawberry anthracnose being a serious

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problem all year round in Taiwan, the control of this disease has become an important topic in strawberry industry currently.

Benefits of biocontrol agents

In the past, farmers mostly used chemical pesticides to control plant diseases in the fields. If we don't use the chemical pesticides more carefully, it will cause the emergence of drug resistant pathogens. On the other hand, since the strawberries are being continuously harvested, we need to carefully medicate during the harvesting season. Otherwise, over applications of the pesticides will lead to residual problems and food safety concerns. Owing to the environmental protection is valued in recent years, less usage of chemical pesticides is promoted. So, how to select non-toxic, safe and efficient replacements of chemical pesticides to control the plant diseases is currently an important topic.

For sustainable agricultural development, biocontrol agent not only must be a potential pesticide for plant protection, but also not be harmful to the environments. The antagonistic microbes can utilize several mechanisms to control the diseases such as antibiosis, lytic enzyme production and induction of systemic resistance. In this study, I aim to figure out the active compounds of the soil microbes which act as antibiotics and then study the molecular mechanisms of how they act against *Colletotrichum gloeosporioides* of strawberry.

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Soil dwelling bacterium: *Photorhabdus luminescens*

Photorhabdus luminescens is a Gram-negative bacterium belonging to the family Enterobacteriaceae and is bioluminescent (Boemare et al., 1993). By 1993, *Photorhabdus* was classified as a new genus separated from *Xenorhabdus* spp. which were symbiotic bacteria of entomopathogenic nematodes (EPN) (Boemare et al., 1993). *Xenorhabdus* and *Photorhabdus* were isolated from infective juveniles of entomopathogenic nematodes Steinernema and Heterobabditis, respectively (Forst, 1997). Entomopathogenic nematodes colonize in the guts with two stages, an infective stage and a free-living stage. The nematodes also utilize the symbiotic bacteria that live in the guts of the nematodes to kill their hosts. When the nematodes enter their hosts, they released the bacteria to kill the hosts during the first 24 hours to 48 hours and took about 7 to 15 days to complete their own life cycles (Daborn et al., 2001). The mechanism of the bacteria-insect interaction is that the nematodes entered the haemocoel of the insect and then secreted several secondary metabolites and enzymes, including lipases, proteases, antibiotic substances, lipopolysaccharides, and high molecular weight toxic complexes (Clarke et al., 1995; Dunphy and Webster, 1988; Schmidt et al., 1988; Wang and Dowds, 1993). So *Steinernema* and *Heterorhabditis* have high potentials in application as biocontrol agents against a range of insect pests (Ehlers, 2010).

On the other hand, *Photorhabdus* and *Xenorhabdus* species have two forms, the

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primary form (phase I) and secondary form (phase II), which can be distinguished by some biochemical tests and physiological properties (Akhurst, 1980; Boemare and Akhurst, 1988). The primary form was isolated from infective juvenile of the nematodes and the secondary form was formed by phase variation or prolonged incubation (Smigielski et al., 1994; Forst, 1997). The primary form has the ability to inhibit a large spectrum of microorganisms because of their antibiotic components (Akhurst, 1982). And two forms have different colony phenotypes on agar media, including nutrient agar (Sharma and Kulshrestha), nutrient agar with 0.004 % (w/v) triphenyltetrazolium chloride and 0.025 % (w/v) bromothymol blue (NBTA) and Macconkey agar (Akhurst, 1980). The primary form has bromothymol blue absorbing ability, viscous colony, pigment of colony production, more antibiotics and protease, while the secondary form does not have these characteristics (Forst and Nealson, 1996; Givaudan et al., 1995; Schmidt et al., 1988). In addition, it was proposed that phase variation is a general mechanism that the bacteria used to adapt to environmental stresses, such as high or low osmolarity, high or low temperature, acid or alkaline condition (Krasomil-Osterfel, 1995).

Secondary metabolites from *Photorhabdus luminescens*

Numerous active compounds have been identified from *Photorhabdus* spp., which contain a wide spectrum of activities against bacteria, fungi, insects and nematodes. *Photorhabdus luminescens* could produce trans-cinnamic acid that is effective against

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pecan scab (Bock et al., 2013). The crude extracts from cultures of *Photorhabdus luminescens* had inhibitory effects on three plant fungal species; *Fusarium oxysporum*, *Alternaria alternate* and *Beauveria bassiana* (Orozco et al., 2016). *Photorhabdus luminescens* could produce isopropylstilbene, which is widespread in planta, but rarely detected in non-plant organisms. Isopropylstilbene has antimicrobial functions and acts as an inhibitor of the insect immune system. However, it can also support normal growth and development in the nematodes (Joyce et al., 2008). Rhabduscin has an inhibitory ability to the insects growth enzyme phenol_oxidase in the insect immune system (Crawford et al., 2012). Glidobactin has antifungal and antitumor cells abilities and acts as a proteasome inhibitor that could protect the insect cadaver (Groll et al., 2008; Oka et al., 1988). Phurealipids acts as an inhibitor of the insects juvenile hormone epoxide hydrolase, which is essential for insect normal development (Nollmann et al., 2015). Photopyrones act as signaling molecules in cell-cell communication to control cell clumping (Brachmann et al., 2013). Lumizinones have inhibitory activities against cysteine protease between the host and the bacteria (Park and Crawford, 2016). Rhabdopeptides also act as insect-specific virulence factors, but the mechanism by which how they act remains unknown (Reimer et al., 2013). The active compounds produced by *Photorhabdus luminescens* are listed in Table 4.

Native *Photorhabdus luminescens* associated study in Taiwan

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In 2004, *Heterohabditis brevicaudis* TG01 was first isolated from soils in Guanzih Mountain of Taiwan by Dr. Feng-Chia Hsieh group (Hsieh et al., 2009). A symbiotic bacterium of *H. brevicaudis* was isolated and identified as *Photorhabdus luminescens* subsp. *akhurstii* based on biochemical tests and 16S rDNA analysis. *Photorhabdus luminescens* subsp. *akhurstii* was renamed to *Photorhabdus akhurstii* in 2018 (Machado et al., 2018). It is worth mentioning that Dr. Hsieh has entrusted the safety-assessment group of Agricultural Chemicals and Toxic Substances Research Institute to do *Photorhabdus* acute toxicity test, the results confirmed that native *Photorhabdus* is s safe bacterium (謝，2010). *Photorhabdus luminescens* subsp. *akhurstii* strain 0813-124 is the main research object of this study. It was shown to have antimicrobial activities against *Glomerella cingulate* （檬果炭疽病）, *Colletotrichum musae* （香蕉炭疽病）, *Penicillium italicum* Pi13 （柑桔青黴病）, *Pestalotiopsis eugeniae* （蓮霧果腐病）, *Botryodiplodia theobromae* （檬果蒂腐病）, *Colletotrichum gloeosporioids* （甜柿炭疽病）, *Rhizoctonia solani* （水稻立枯絲核菌）, *Sclerotium rolfsii* Saccardo （百合白白絹病）, *Botrytis elliptica*（百合灰黴病）, *Botrytis cinerea*（玫瑰灰黴病）, *Alternaria mali* （蘋果褐斑病）, *Aspergillus niger* （洋蔥黑趨病）, *Fusarium solani* （荔枝鐮胞菌）, *Fusarium solani*（蘭花鐮胞菌）, *Fusarium oxysporum* f. sp. pisi（碗豆鐮胞菌）, *Fusarium oxysporum* f. sp. lycopersici（番茄鐮胞菌）and *Phytophthora capsicii*（甜椒疫病） (謝，2010).

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In addition, it was found that the strain 0813-124 has activities against *Micrococcus luteus* BCRC 10449 in 2008. Further, they used low osmolarity to induce the switch from phase I to phase II. They also found that the bioluminescence, chitinase and protease produced during phase I were higher than during phase II. Then, they injected 0813-124 to *Galleria mellonella* and cultured for 5 days. After purification (acetone extracts, TLC and HPLC) and identification (LC Q-TOF MS), the active compounds of 0813-124 was similar to 3,5-dihydroxy-4-isopropylstilbene (王，2008).

However, there is no information on whether the native strain 0813-124 could produce active compounds that can act against phytopathogens in Taiwan.

Syrbactins biosynthetic mechanism

Syrbactins are cyclic peptide derivatives and consist of a 12-membered dipeptide-macrolactam which are known to inhibit the eukaryotic proteasomes (Dudnik et al., 2013). Syrbactins consist of 4 groups of compounds, including syringolins, glidobactins, cepafungins and luminmycins (Krahn et al., 2011). These compounds are synthesized by nonribosomal peptide synthetases (NRPS) and polyketide synthase (PKS). NRPS is a large module enzyme that can activate and condense amino acids via a thiotemplate mechanism.

On the other hand, cepafungin I-III and glidobactin A-G have been identified (Oka et al., 1988; Shoji et al., 1990). Cepafungin was the antibiotics first isolated from

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Burkholderia cepacia culture broth and showed inhibitory activity against yeast, fungi and antitumor activity in P388 leukemia of the mice (Shoji et al., 1990). The fungi that were inhibited by the cepafungin complex include *Candida albicans*, *Aspergillus fumigatus*, *Penicillium digitatum* and *Microsporum canis*, based on the bioassays that were measured on Sabouraud's glucose. And glidobactin was first isolated from *Polyangium brachysporum* sp. nov. No. K481-B101 with antifungal and antitumor activities (Oka et al., 1988).

Significance of this study

The long-term goal of this project is establish a platform for purification and identification of active compounds produced by *Photorhabdus akhurstii* sp. nov 0813-124. The main purpose is to explore this bacterium to identify biocontrol pesticides with antifungal and anti-insects activities and determine the mechanisms of their actions. In addition, this study will focus on active compounds produced by this native species that can act against *Colletotrichum gloeosporioides* of strawberry in Taiwan.

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Materials and Methods

Bacterial strains, culture media and growth conditions

Pseudomonas taiwanensis sp. nov. CMS^T (=BCRC17751^T=DSM21245^T) was obtained from Dr. Wen-Jen Chen (Chen et al., 2016). Antagonistic assay of *P. taiwanensis* against *Colletotrichum gloeosporioides* was tested on Potato dextrose agar (PDA) plate at 28 ℃. *P. taiwanensis* was pre-cultured in LP medium in 125 mL flask at 30℃ for 16 hours.

Bacillus amyloliquefaciens strain ASB111 was isolated from soil in 北溝試驗田 of National Chung Hsing University, Taichung by Dr. Wen-Jen Chen. Antagonistic assay of ASB111 against *Colletotrichum gloeosporioides* was tested on PDA plate at 28 ℃. ASB111 was pre-cultured in LB in 250 mL flask at 30℃ for 16 hours. The loading volume of each hole was 30 μL.

Photorhabdus akhurstii sp. nov. 0813-124 was provided by Dr. Feng-Chia Hsieh. *P. akhurstii,* designated PL, which was isolated from an entomopathogenic nematode of the family Heterorhabditidae in Tainan (Hsieh et al., 2009). To set up stable growth conditions of PL, I prepared six different liquid media, including LP, LP, NB, PP3T, TSB and PDB (The composition of each medium is listed in Table 3). I also checked on which medium PL could have strong antagonistic activity. For antagonistic assays, PL was pre-cultured in LP liquid medium in a test tube containing 3 mL medium and incubated at 30℃ and

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200 rpm for 16 hr. Then, MacConkey agar was used (see Table 3 for medium composition) to do streak-plate for 2 days. Single colonies (around 0.2 cm) were picked and cultured in LP liquid medium and incubated in a 125 mL flask containing 25 mL medium at 30℃ and 200 rpm for each experiment.

Replica plating

For replica plating, 200 μL of PL1 culture from 24 hr culture broth was streaked out on LB plate as a master plate and the velvet was pressed on the LB agar. The bacterial colonies were transferred by the block to the PDA plates.

Fungal growth conditions

Colletotrichum gloeosporioides (Cg) strain used in this study, which was isolated from the strawberry, was provided by Dr. Feng-Chia Hsieh, Biopesticide Division, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council of Agriculture. Other *Colletotrichum* spp. were provided by the Department of Plant Pathology, National Chung Hsing University. Fungi were pre-cultured on PDA medium and incubated at 28℃ for 7 days. Then fungal agar disks were used to do further antagonistic assays.

For spores-germinated-plates, the five days Cg growth plates were collected and the sterilized water was used to wash the spores on PDA. The glass rod was used to wash the cultures. The mesh was taken to filter the filtrates. The suspensions were centrifuged and

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formulated for further experiments. The concentration of spores-germination-plate was ~10^5 spores/mL.

Antagonistic assays

For antagonistic assays on plates, 90 μL of bacterial cultures and Cg cultures were injected separately into the holes at different locations of the PDA plates. The bacterial broth and Cg were put on PDA at the same time. The measurement of inhibition zones was started after 4 days incubation.

For comparison of antagonistic assays on different solid media, PDA, LPA, ½ (LP+PDB) agar and (1/4LP+3/4PDB) agar were prepared. 90 μL of bacteria cultures were also injected into the hole of the Cg agar disks on the PDA plate until the inhibition zone has been measured.

Colony-forming unit (CFU) test

Each culture broths were done serial dilution, then the diluted broths were added 100 μL to NA plate. Then the plates incubated at 30℃ for 2 days and calculated by cytometer.

Active compounds extraction methods

For extraction of active compounds from ASB111, ASB111 was cultured in two 250 mL flask for three days and 100 mL of ASB111 cultures were used to do antagonistic assays (30 μL/ hole on PDA plates, in~40 PDA plates) at 30℃. After bioassays, the agar of the bacteria culture and inhibition zones area were cut and extracted with organic

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solvent with a volume sufficient to flood the agars for 16~18 hrs. The organic phase was evaporated under reduced pressure to dryness at 30℃ by a rotary evaporator. The crude extracts were weighted and suspended in 0.1% DMSO or 50% (0.1%DMSO+MeOH) and filtered with a 0.22 μm PVDF membrane. In order to get higher extraction efficiency, I also compared different polarity organic solvents, including 1-butanol and ethyl acetate (EA).

For PL1 active compounds extraction, I examined three extractions, including supernatant extraction, cell-disruption extraction and cut-agar extraction.

For supernatant extraction, PL1 was cultured in LP medium in a 125 mL flask at 30℃ for 5 days and centrifuged to get rid of pellets. The supernatants were centrifuged and filtered with a 0.22 μm PVDF membrane. The filtrates were extracted with equal volume of ethyl acetate for 16~18 hr. The organic phase was evaporated under reduced pressure to dryness at 30℃ by a rotary evaporator. The dried filtrates were weighted and resuspended in methanol and filtered with a 0.22 μm PVDF membrane. The final filtrates were used for further bioassays and characterization. Second cell-disruption extraction, PL1 was cultured in LP medium in a 125 mL flask at 30℃ for 5 days and the cultured broth was disrupted by a desktop high pressure homogenizer. The cell disrupted broth was centrifuged and got rid of pellet. The supernatant was filtered with a 0.22 μm PVDF membrane. The filtrate was extracted with equal volume of ethyl acetate for 16~18 hr.

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The organic phase was evaporated under reduced pressure to dryness at 30℃ by a rotary evaporator. The dried filtrates were weighted and resuspended in methanol and filtered with a 0.22 μm PVDF membrane. The final filtrates were used for further bioassays and characterization.

For cut-agar extraction, PL1 was pre-cultured in LP medium in 125 mL flask at 30℃ for 24 hr and then transferred to PDA plates (90 μL / hole on PDA plates) to do antagonistic assay. After bioassays, forty agar plates were cut and extracted with equivalent volume of organic solvent to flood the agars for 16~18 hrs. The organic phase was evaporated under reduced pressure to dryness at 30℃ by a rotary evaporator. The residual undried crude extracts were transferred to 2 mL tubes and evaporated to dryness by a refrigerated CentriVap Concentrator. The dried extracts were weighted and resuspended in methanol and filtered with a 0.22 μm PVDF membrane. The final filtrated were used for further bioassays and characterization. In order to get higher extraction efficiency, I also compared organic solvents with different polarities, including 1-butanol (BuOH) and ethyl acetate (EA). The crude extracts concentration of three methods for further experiments were 100 mg / mL.

HPLC

ASB111 crude extracts were separated by reverse-phase high-performance-liquid-chromatography (HPLC) of Agilent Technologies 1100. The samples were loaded onto a

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Supelco Discovery HS C18 column (25 cm × 10 mm, 5 μm particle diameter). The mobile phase components were H_2O and ACN. The fractions were eluted at a flow rate of 1 mL/ min with a gradient condition of ACN, developed from 5% to 100%. The separation pattern was monitored by determining absorbance at 210 nm, and each fractions were collected by an auto-collector, used for bioassays and were identified by LC-MS/MS.

PL1 crude extracts were separated by reverse-phase by high-performance-liquid-chromatography (HPLC) of Agilent Technologies 1100. The samples were loaded on a Supelco Discovery HS C18 column (25 cm × 10 mm, 5 μm). The mobile phase components were H_2O and ACN. The fractions were eluted at a flow rate 2.5 mL/ min with an isocratic condition of ACN 50% in 60 mins with a flow rate of 2.5 mL/ min. The separation pattern was monitored by determining absorbance at 270 nm, and each fractions were collected by an auto-collector, and used for bioassays and were identified by LC-MS/MS. The detector of this LC system is DAD, the separation of the sample was based on the absorption wavelength. On the other hand, if we want to quantify the area of the active peak real content, the best choice is CAD detector of HPLC.

Analysis of active compounds from ASB111 by Mass spectrometry

LC-MS/MS (LCQ)

ASB111 1-butanol extracts and active fraction from HPLC were purified and identified by LC-MS/MS. The 1-butanol extracts analyzed concentration was 30 mg/ mL and

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injected 5 mL. The LC system was Aglient 1100 HPLC Phenomenex Luna C18(2) 3 μm (150 X 2 mm), the separated flow rate was 0.2 mL/ min, the column temperature was 25 ℃. The active compounds were separated by applying an acetonitrile in H_2O gradient from 0% to 100% in forty minutes. The mass system was Thermo LCQ (Ion-trap) and positive ions mode of ESI.

Analysis of active compounds from PL1 by Mass spectrometry

LC-MS/MS (Orbitrap)

For LC-MS/MS analysis, the crude extracts were concentrated to dryness by a rotary evaporator at 30℃ and then dissolved in MeOH for LC-MS/MS analysis. The C18 column (Water UPLC ACQUITY UPLC BEH C18 1.8 μm, 100 × 2.1 mm) was used with the following gradients: 0~6 min at 5%~99.5% of ACN+0.1% FA, 6~8 min at 99.5% of ACN+0.1% FA, 8~8.2 min at 99.5%~5% of ACN+0.1% FA and 8.2~10 min at 5% of ACN+0.1% FA. The rate of the flow was 0.4 mL/ min. The mass data were analyzed by the Thermo Orbitrap Elite system and the profile mode was set on positive mode with mass range *m/z* 50~1500. And the resolution of Orbitrap were 60000 resolution (MS1), 15000 (DDA) and 30000 (DDA2). For tandem mass (MS/MS), the top five intense ions from each full mass scan were selected for collision-induced dissociation fragmentation (CID). Then the fragments from MS/MS data can be used to predict the structures.

High resolution mass spectrometry (HRMS)

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High resolution and high mass accuracy experiments were done on a LTQ Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with standard ESI ion source. 5 μL of sample were injected at a flow rate of 50 μL / min in 80% ACN/ H_2O 0.1%FA by Ultimate 3000 RSLC system from Dionex (Dionex Corporation, Sunnyvale, CA). Full-scan MS condition: mass range *m/z* 100-2000, resolution 60,000 at *m/z* 400. The target ions were sequentially isolated for MS2 by LTQ. Electrospray voltage was maintained at 4 kV and capillary temperature was set at 275 ºC. High resolution mass spectrometry analyses were performed by GRC Mass Core Facility of Genomics Research Center, Academia Sinica.

RNA extraction

TRIZOL extraction

For RNA extraction of liquid culture broth and agar incubated plate, 2 ml culture broth were centrifuged for 10 min at 8000 xg at 4℃ and the supernatants were removed. Total RNAs were extracted from 2 ml pellets by adding 500 μL of TRIZOL™ (Invitrogen, Carlsbad, CA, USA) reagent. The pellets were vortexed with TRIZOL reagent and placed at room temperature for 5 min. Next, added 100 μL chloroform (1/5 of TRIZOL volume) to the tube and shook it, followed by incubating at room temperature for 3 min. The samples were centrifuged for 15 min at 12000 xg at 4℃, and transferred the supernatants to new 1.5 mL tubes. Adding 250 μL isopropanol to deposit the samples, placed at room

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temperature for 5 min and mixed them well. The samples were centrifuged at 12000 xg at 4℃ for 10 min. After centrifugation, the supernatant was removed and the pellet was washed with 1 mL 75% ethanol. The tubes were vortexed to detach the pellets from the tube wall and centrifuged for 5 min at 12000 xg at 4℃. The pellet was washed by 75% ethanol twice and 99% ethanol once and then for 5 min at 12000 xg at 4℃. The supernatants were removed and the pellets air dried for 20 min. Twenty μL RNase free water was added to dissolve the pellets. The concentrations of RNAs were measured with Nanodrop (ND-10000 spectrophotometer).

DNase treatment

For DNase treatment, 20 μL samples were treated with 2 μL TURBO DNase I and incubated at 37℃ for 30 min and 1/10 volume of DNase Inactivation reagent was added and mixed well. The reaction mixtures were incubated at room temperature for 2 min with occasional mixing. Afterward, the samples were centrifuged at 12000 xg for 2 min at 4℃. The upper supernatants were transferred to a fresh tube. The concentration of RNAs was quantified with Nanodrop (ND-10000 spectrophotometer).

Reverse transcription

After qualification, 1 μg total RNA was added with 2 μL random primer (250 ng/μL) and 1 μL 10mM dNTP. The sample mixture was denatured at 70℃ for 10 min in a PCR machine, followed by chilling at 4℃ (on ice) for 5 min immediately. To generate cDNA,

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4 μL of 5X first strand buffer, 2 μL 10 mM Dithiothreitol (DTT), 0.5 μL 20 unit of ribonuclease inhibitor (RNase OUT), 0.5 μL RNase-free water and 1 μL RNA Moloney murine leukemia reverse transcriptase (M-MLV RT) were added to the denatured RNAs and incubated 37℃ for 1 h. The reaction was stopped at 65℃ for 10 min (PCR machine). Finally, the resulting 20 μL cDNA mixture was diluted with 80 μL RNase-free water.

Quantitative RT-PCR

For quantitative RT-PCR, the 2 μL diluted cDNA product was mixed with 7.2 μL of ddH_2O, and then 0.4 μL both of forward and reverse specific primers, 10 μL SYBR Green Master Mix was added. After mixing well, quantitative RT-PCR measurement was performed by a Q-PCR detection system (ABI 7300 Real-Time PCR machine). The program set up was 40 cycles of 15 sec at 95℃, 60sec at 60℃. The data analyzed with the 7300 System SDS software, and the delta C_T (cycle of threshold) was detected to determine the relative expression level of genes. The delta C_T value of target gene was further normalized to the housekeeping gene, 16S, which was used as an internal control.

Omic profiles

The whole genome sequencing was done by Dr. Feng-Chia Hsieh and analyzed by Dr. Chih-Cheng Chien. RNAseq and transcriptomic profiles were performed by Dr. Chih-Cheng Chien.

AntiSMASH software

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The AntiSMASH web server (https://antismash-db.secondarymetabolites.org/) was used as for automatic genomic identification and analysis of biosynthetic gene clusters. This study used version 5.0 to predict the metabolites of PL.

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Results and discussion

Screening of soil microbes for bacteria with antagonistic activities against *Colletotrichum gloeosporioides*

We have designed an antagonistic assay to screen soil microbes for potential bacteria with antagonistic activities against *Colletotrichum gloeosporioides* (Cg). Four bacteria, including *Pseudomonas taiwanensis* sp. nov. CMS^T (Pt), *Bacillus amyloliquefaciens* strain ASB111 (ASB111), *Photorhabdus akhurstii* sp. nov. 0813-124 phase I (PL1) and *Photorhabdus akhurstii* sp. nov. 0813-124 phase II (PL2) were tested. The information of bacteria was listed on Table 2. After 7 days, the results showed that Pt did not have anti-Cg ability (Figure 1), but ASB111 had better anti-Cg ability (Figure 1). Interestingly, PL1, but not PL2, had anti-Cg ability, although they are different phases of the same bacterium (Figure 1). Although ASB111 and PL1 had the anti-Cg ability, they had different effects on the features of the hyphae of Cg in my observation. The antagonistic ability of Cg by ASB111 resulted in tight square morphology of Cg (Figure 1). The antagonistic ability of PL1 also made Cg to square shape, but the edge appeared to be filamentous hyphae (Figure 1).

Active compounds of ASB111 1-butanol extracts were expected to be iturin, fengycin and surfactin

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Based on the effectiveness of ASB111 on the antagonistic plate assays, I expected the active compounds of ASB111 were different from supernatant extraction and cell-disruption extraction. Therefore, I used the cut-agar method to do further extraction. In order to get better extraction efficiency, I compared two organic solvents with different polarities, including 1-butanol and EA. The results indicated that the 1-butanol extracts of ASB111 had the better inhibitory ability on spores-germinated-plates (Figure 2-1). Therefore, 1-butanol is the better organic solvent to extract active compounds of ASB111.

Further, I used high-performance-liquid-chromatography (HPLC) to analyze 1-butanol extracts. 1-butanol extracts were separated by C18 column and each fractions were subjected to do antagonistic assays. I found that the 29th and 30th fractions had anti-Cg ability (Figure 2-2-A). Further, I prepared 1-butanol extracts and 29th fraction to the Metabolite Core Lab of ABRC to identify the active compounds. After LC-MS analysis, the MS^1 spectra showed a stronger signal, I assumed that active compounds of this signal of the 29th fraction was iturin (*m/z* 1043) (Figure 2-2-B). I also analyzed 1-butanol extracts by LC-MS. Based on MS spectra, I deduced that the active compounds of ASB111 are likely to consist of iturin, fengycin and surfactin (Figure 2-2-C), which were previously identified in studies of other *Bacillus* species (Kim et al., 2010). Therefore, I decided to focus my study on a novel native bacterium, *Photorhabdus akhurstii* sp. nov 0813-124, which has not been reported to have anti-Cg activities until now.

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Strategies of active compounds produced by *Photorhabdus akhurstii* sp. nov 0813-124

The strategy I used to study of *Photorhabdus akhurstii* sp. nov 0813-124 (PL) is outlined in Figure 3. I first tried set up a stable culture condition of PL. After I figured out which medium was suitable and stable, I started to find the better extraction condition to isolate active compounds of the best culture condition. Next, I used mass spectrometry to do analysis and characterization, including LC-ESI-MS (Orbitrap), MALDI-TOF-MS, HPLC combined HRMS, and MALDI-Imaging-MS. LC-ESI-MS (Orbitrap) and MALDI-TOF-MS were used to identify the probable compounds in PL1 and PL2. EA extracts I prepared included extracts from LPA, PDA, PL1/PDA, PL1/LPA, PL2/PDA and PL2/LPA. The LC-ESI-MS and MALDI-TOF/MS data were used as PL1 and PL2 active compounds database and for further compounds comparison. For analysis of antagonistic scale of secreted compounds, I did MALDI-Imaging MS. On the other hand, I combined genomic data and transcriptomic profiles to do genetic analysis to find out the biosynthetic genes of target metabolites.

PL could not grow in PDB medium

For set up PL1 stable culture condition, I screened several liquid media to determine

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which could support PL growth and release active compounds against *Colletotrichum gloeosporioides*. Six liquid media I selected, including LB, LP, NB, PDB, PP3T and TSB, and the ingredients of each medium are listed on Table 3. The characteristics of these media are as follows: First, LB is a general medium. Second, LP is an iron-limited medium and was used on *Pseudomonas taiwanensis* study for efficient production of antimicrobial compounds (Chen et al., 2016). Third, NB is a basic medium composed of simple peptone and beef extract. Fourth, PDB is a fungal growth medium composed of potato extracts and dextrose. Fifth, PP3T was used for *Photorhabdus luminescens* strain ATCC 29999 to produce toxin complex (Tc) to against insects (謝等，2004). Sixth, TSB was modified to 1/2 TSB on *Pseudomonas taiwanensis* MALDI-Imaging MS analysis (Chen et al., 2016). I observed that both PL1 and PL2 could not grow in PDB medium after 24hr culture time (Figure 4-1-A). To confirm this observation, I transferred colonies of PL1 and PL2 grown LB agar plates to PDB agar by replica plating. The results also showed that PDA as replica plates did not support PL1 and PL2 to grow on (Figure 4-1-B). Besides, I also observed that PL still did not have activity in PDB even after it had grown for 120hr. Hence, I deduced that potato extracts and the dextrose of PDB were not suitable for the growth of PL1 and PL2. On the other hand, I discovered that the main ingredients of LB, NB, PP3T and TSB are tryptone or peptone, then I designed 1/2PDB+1/2PP3 to verify whether peptone is a key factor to affect the activity of PL. The

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results indicated that PL can grow in 1/2PDB+1/2PP3 medium (Figure 4-1-C). And the cultures of 1/2PDB+1/2PP3 showed antagonistic activity (Figure 4-1-C).

PL1 LP cultures had stronger activity to inhibit Cg at 24hr culture time

To investigate the optimal culture time for PL1 to release maximal levels of active compounds against Cg, I examined several media, including LB, LP, NB, TSB and PP3T to do further antagonistic assays. I selected three time-points, 24 hr, 72 hr and 120 hr as culture times and used each time-points cultures for antagonistic assays. The results showed that the antagonistic abilities of the 24 hr and 72 hr cultures were significantly higher than that of 120 hr cultures (Figure 4-2-A). Among different media, it was worth noting that PL1 showed significantly anti-Cg activity when it was grown in LP medium (iron-limited medium) and TSB at 24 hr culture time (Figure 4-2-B). The antagonistic activity of TSB cultures was declined 50% via 120 hr continuous culture and LP cultures was only declined 33% via 120 hr continuous culture (Figure 4-2-B). Therefore, LP medium is a stable and active medium to culture PL1. Intriguingly, LP medium is nutrient-limited and it just composed of several salts, such as $Na_2HPO_4 \cdot 7H_2O$, KH_2PO_4, NaCl and NH_4Cl. However, many *Pseudomonas* strains were cultured in this iron-limited medium (LP medium) to produce active compounds pyoverdine, which acts as an iron-chelator in the cell signals transport process (Chen et al., 2016). Thus, I proposed the salts and limited

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nutrients from the LP medium might be a stress condition to induce PL1 active compounds against Cg at 24 hr culture time.

Next, I performed experiments to determine whether the antagonistic activities are directly proportional to the PL1-colony-forming unit (CFU), i.e. cell numbers. The results suggest that the antagonistic activities produced by PL1 were not correlated with CFUs in each medium. First, I found that PL1 had the highest CFU at the 24 hr cultures in NB and TSB (Figure 4-2-C). However, PL1 had the highest CFU at 72 hr in NB and PP3T (Figure 4-2-C). After 120 hr, CFU declined in all media (Figure 4-2-C). At the same time, I observed the characteristic of the PL1 culture broth color. LB, PP3T and TSB cultures had turned black after 72 hr, I supposed that CFU variation of these media might be associated with blackened broths (Figure 4-1-A). However, these culture broths contained both live bacteria and dead bacteria at the same time which led to PL1 still had the anti-Cg function at 120 hr cultures (Figure 4-2-A). However, I found that the media had not blackened in the PL2 cultures. Taken together, PL1 had the best antagonistic ability in the LP medium at the 24 hr culture time (Figure 4-1-A, 4-2-A) and that the CFU of LP medium indicated that PL1 relied on less CFU to release active compounds against Cg at 24 hr culture time (Figure 4-2-C). Otherwise, LP medium is nutrient-limited, so that PL1 cultures grow slowly to induce the antagonistic ability on plates.

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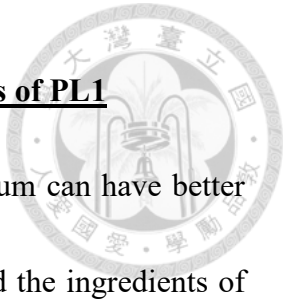

PDB agar (PDA) might act as a stress to induce active compounds of PL1

Figure 4 and previous data showed that PL1 cultured in LP medium can have better activity. So I decided to investigate whether solid medium contained the ingredients of LP could support or make PL1 release more active compounds. I designed four kinds of solid media, including PDA, LPA, 1/2LPA+1/2PDA and 1/4LPA+3/4PDA. Interestingly, I found that PDA was the only solid medium that can induce PL1 to release active compounds that had anti-Cg ability (Figure 4-2 D). It was so interesting that PL1 cannot grow in the PDB liquid medium, but PL1 pre-cultured in LP medium and then transferred to the holes of PDA solid medium can make PL1 produce more active compounds against Cg. I have already known that PL1 cannot use potato extracts and dextrose of PDB for growth, but PDB agar (PDA), which has limited nutrients, might play a role as a stress to induce the production of active compounds in PL1 to inhibit Cg. However, the solid media containing the ingredients of LP did not show better antagonistic activity between PL1 and Cg (Figure 4-2-D).

Cut-agar method with EA extracts of PL1 incubated in PDA has best efficiency

To find the most efficient method for the extraction of active anti-Cg compounds from PL1, I tested three kinds of extraction methods with EA, including supernatant extraction, cell-disruption extraction and cut-agar extraction. First, the results of the

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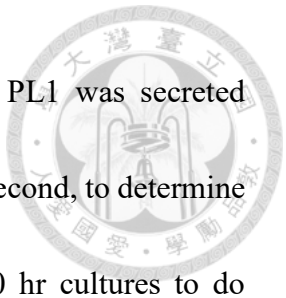

antagonistic plate assays suggested that the active compounds of PL1 was secreted extracellularly, I used the supernatants of 120 hr cultures to extract. Second, to determine whether the active compounds remain intracellular, I used the 120 hr cultures to do pressure cell-disruption for extraction. Third, previous data showed PDA might induce active compounds of PL1 on antagonistic assay, so I decided to use the cut-agar method to extract active compounds from the 96 hr incubated plates (PL1 cultures were pre-cultured in LP medium for 24 hr). After comparison of three kinds of extraction methods, the results showed that the cut-agar method had the best extraction efficiency with 1 cm inhibition zone after 2-days-spores-germinated-plates (Figure 5A). However, the supernatants extraction and cell-disruption extraction just had 0.55 cm inhibition zone and 0.45 cm inhibition zone, respectively (Figure 5A).

On the other hand, I was curious about whether organic solvents with different polarities had different extraction efficiencies. Therefore, I used EA and BuOH to extract agars and prepared condensed extracts (10 mg/mL) as described in the Materials and Methods. Then, I tested six combinations, including PDA cultures of EA extracts, PDA cultures of BuOH extracts, 1/2EA extracts plus 1/2BuOH extracts with PDA cultures, LPA cultures of EA extracts, LPA cultures of BuOH extracts and 1/2EA extracts plus 1/2BuOH extracts with LPA cultures.

After 2-days-spores-germinated-plates incubation, I found that LPA cultures of EA

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extracts and BuOH extracts had similar inhibitory ability to Cg (Figure 5C). The previous antagonistic assay showed the ingredients of LP medium could not support PL1 to have better antagonistic activity. Besides, I compared PDA cultures of EA extracts and BuOH extracts. EA extracts indicated highest efficiency against Cg (Figure 5B). The inhibition zones were still maintained even if spores-germinated-plates were incubated for 6 days (Figure 5E). In addition, I also found that BuOH extracts did not have added effects on the inhibitory ability of EA extracts (Figure 5D, G). On the other hand, to verify that PDA might be a stress to induce more active compounds production of PL1, I compared PDA cultures and LPA cultures with EA extracts. On 2-days-spores-germinated-plates, LPA cultures still had inhibitory activity (Figure 5C). However, after 6 days of incubation, the inhibition zone of LPA cultures was closed (Figure 5F), but PDA cultures still had inhibition zone (Figure 5E).

Taken together, these results suggested that EA is the best organic solvent to extract active compounds of PL1 and PDA really induced more active compounds of PL1, but LPA extracts also had inhibitory activity. Next, I set up reactions to determining the minimum inhibitory concentration of PL1 EA extracts. I used 50 mg/mL as the initial concentration and diluted to 25 mg/ mL, 12.5 mg/ mL, 6.25 mg/ mL, 10 mg/ mL, 1 mg/ mL and 0.1 mg/ mL. The antagonistic assays showed that 1 mg/ mL of EA extracts was the minimum inhibitory concentration on 2-days-spores-germinated-plates (Figure 5I).

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The concentration under 1 mg/ mL gradually lost anti-Cg activity.

The active compounds of PL1 fractions which separated by HPLC is cepafungin I

Next, I used HPLC to analyze EA extracts of PL1. 50 μL of 100mg/ mL EA extracts were injected and separated by the C18 column. The active fractions eluted from RT 12 min to 16 min had anti-Cg ability by bioassays under our separated conditions (Figure 6A). I found that the peak absorbed wavelength was around 270 nm. Among them, the concentration of ACN was around 50% and the seventeenth fraction had stronger anti-Cg ability (Figure 6A). Therefore, it is likely that active compounds of PL1 were in this fraction. Further, I performed high resolution mass spectrometry (HRMS) in GRC Mass Core Facility to identify the active compounds of the seventeenth fraction. The active signal of MS^1 spectra with mass-to-charge (*m/z*) ratios was *m/z* 535.35 (Figure 6B) and based on tandem mass spectrometry (MS^2 fragmentation profiles), the corresponding compound was predicted to be cepafungin I (Figure 6C). In addition, we also found other similar derivatives in PL1 extracts of LC-ESI-MS data. The signals of similar derivatives were *m/z* 521 and *m/z* 549, which were subjected to tandem mass spectrometry (MS^2 fragmentation profiles) for compounds analysis and structure prediction. The compounds of *m/z* 521 and *m/z* 549 were glidobactin A and glidobactin C, respectively (Figure 6F). Cepafungin I, glidobactin A and glidobactin C belong to the same family compounds of

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syrbactins, which are microbial natural products (Krahn et al., 2011). To the best of my knowledge, this is the first study to discover that cepafungin I, glidobactin A and glidobactin C had antagonistic activity toward plant fungal disease. The structures of these compounds were drawn and predicted by Dr. Yu-Liang Yang. To get the more exact structure of these compounds in PL, we need to do the NMR analysis.

Syrbactins might be highly induced by PDA incubation

It may be noted that I have observed special characteristics of syrbactins compounds in different extractions. I discovered that syrbactins compounds were highly extracted by cut-agar method (Figure 6E). The supernatant extraction and the cell-disruption extraction showed much lower syrbactins signals in LC-MS spectra (Figure 6E). Then, based on cut-agar extraction, I found that when PL1 and PL2 sub-cultured on LPA for extraction, syrbactins compounds (*m/z* 521, *m/z* 535 and *m/z* 549) were just observed in PL1 LC-MS spectra (Figure 6D). On the other hand, I observed that when PL1 and PL2 were sub-cultured on PDA for extraction, both of PL1 and PL2 produced glidobactin A (*m/z* 521). But PL1 also had cepafungin I (*m/z* 535) and glidobactin C (*m/z* 549) (Figure 6D).

These results can be linked to our previous results that PDA act as a stress to induce more active compounds production. Based on antagonistic assay results of PL1/PDA EA extracts and the seventeenth fraction, I proposed that PL1 could not only rely syrbactins

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of EA extracts to suppress Cg. Because of PL1/PDA EA extracts had stronger anti-Cg activities on 2-days-spores-germinatied-plates than the seventeenth fraction (Figure 6A, 5B). So, I considered that anti-Cg ability of the active compounds in PL1 EA extracts were synergistic complex. However, syrbactins compounds were highly induced by PDA incubation and play a role in enhancing the anti-Cg ability of PL1 EA extracts.

The probable mechanisms of syrbactins based on genome analysis and MS results

To investigate the biosynthetic pathways of syrbactins, I used anti-SMASH software to analyze genome sequences of PL1 and PL2. The anti-SMASH software was developed to identify putative biosynthetic pathways for natural products based on genomic sequence data and metabolite profiling. After anti-SMASH (5.0 version) analysis, I found that there were 22 biosynthetic gene clusters (BGCs) in PL1 and PL2, and the most similar metabolites of PL are the same (Figure 7A).

Among them, I had known that syrbactins consisted of syringolins, glidobactins, cepafungins and luminmycins. The anti-SMASH analysis showed that the metabolites prediction of region 8 was including 100% of the genes having similarity to luminmycin biosynthetic genes, 26% of genes show similarity to glidobactin and 100% of genes show similarity to isopropylstilbene (Figure 7B). Some biosynthetic genes of this gene cluster belonged to NRPS (non-ribosomal peptide synthetase) and (PKS Polyketide synthase)

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type. It is worth noting that the gene cluster had 26% of genes having similarity to gilidobactin biosynthetic genes (Figure 7B). These 26% cluster region contained core biosynthetic genes (*09520* and *09530*), additional biosynthetic genes (*09515*), transport-related genes (*09525*) and other genes (*09535*). Further, I supposed that cepafungin I, glidobactin A and glidobactin C of syrbactins family were synthesized by the core biosynthetic genes (*09520* and *09530*) of this cluster. To verify our hypothesis, I did qRT-PCR to confirm the gene expression of *09520* and *09530*. I used the 24 hr LP liquid cultures, 24 hr LPA cultures, 60 hr LP liquid cultures and 60 hr LPA cultures to do RNA extraction. Based on liquid incubation RNAs extraction, PL1 did not have highly transcript levels in core biosynthetic genes, *09520* and *09530* (Figure 7D). But The RT-PCR results indicated that that *09530* might affect biosynthetic process based on agar incubation RNAs extraction (Figure 7E). PL1 had higher transcript levels than PL2 of *09530* at 24 hr and 60 hr agar cultures. I assumed that this fold-change might be similar to mass data that syrbactins were highly extracted by agar incubation. To do further study of syrbactins compounds, using RNAseq to perform to transriptomic analyses might be able to identify additional metabolite related genes that have not been predicted until now.

On the other hand, I observed that region15 still had other biosynthetic genes (*16525*, *16480* and *16485*), with 15% of genes showing similarity to glidobactin biosynthetic genes (Figure 7C). All of these genes might be related to the biosynthesis of glidobactin.

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Genomic profile and transcriptomic analysis

Based on antagonistic assay data, I found that PL1 had anti-Cg ability, but PL2 did not. Dr. Feng-Chia Hsieh had already done whole genome sequencing of these two phases and genome assemble and analysis were done by Dr. Chih-Cheng Chien. Besides, the RNA-seq profile also was finished by Dr. Chih-Cheng Chien, the RNA-seq was done with 24 hr cultures.

Then, I combined the genomic profile and transcriptomic data to do the chart, the chart showed that several genes had different expression profiles such as those in region 8, 10 and 21 (Figure 7A). The chart can serve as a database to assist us to predict biosynthetic genes of active compounds. It is worth noting that the active compound of region 10, isopropylstilbene has been reported to have antagonistic activity against plant fungal pathogens, including *Colletotrichum lagenarium* (Kumar and Nambisan, 2014; Jian et al., 2015; Shi et al., 2017). However, I have not isolated stilbene under my screening process.

Furthermore, the genome structure (done by Dr. Chih-Cheng Chien) showed that a chromosomal segment containing 20 genes in the PL1 genome was deleted in the PL2 genome (Figure 8A). Among these genes, *CE143_11430*, which was annotated as encoding chitinase, had the highest reads of transcripts (Figure 8A). However,

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transformation of this gene into PL2 (performed by Dr. Chih-Cheng Chien), did not restore the antagonistic ability of the PL2 transformants (Figure 8B). Therefore, the chitinase was not a factor to affect antagonistic ability. To confirm whether *CE143_11430* was transformed into PL2 successfully, I did RT-PCR to do examination. The results showed that PL2 tranformants had a higher transcript level of *CE143_11430* than those of PL1 and PL2 (Figure 8C).

PL1 had the activity against *Collectotrichum* spp.

Additionally, we had some strains of *Collectotrichum* genus, including *Colletotrichum acutatum* 153 (Pepper), *Colletotrichum acutatum* 365 (Pepper), *Colletotrichum acutatum* 524 (Pepper), *Colletotrichum gloeosporioides* Tyc2 (Mango) and *Colletotrichum higginsianum* Pa01 (Pai-tsai), which were provided by Department of Plant Pathology, National Chung Hsing University. And *Colletotrichum gloeosporioides* which caused strawberry anthracnose was provided by Dr. Feng-Chia Hsieh (Table1). I first observed that different strains of the *Collectotrichum* spp. had different growth rates, so I did not quantify the results. Based on the antagonistic assay results, I determined that PL1 may be a potential biocontrol agent that had antagonistic activities against a broad range of *Collectotrichum spp* (Figure 9).

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Conclusions and perspectives

The antagonistic assays showed that *Bacillus amyloliquefaciens* strain ASB111 and *Photorhabdus akhurstii* sp. nov. 0813-124 phase I (PL1) had the potential as biocontrol agents against *Colletotrichum gloeosporioides* on strawberry. The active compounds produced by ASB111 had been previously reported (Kim et al., 2010). Further, my study of *Photorhabdus akhurstii* sp. nov. 0813-124 revealed that PL1, but not PL2 could inhibit the growth of *Colletotrichum* spp. pathogens. PL1 from 24 hr cultures in the LP medium (iron-limited medium) had the best antagonistic activity and required less CFU to have the best antagonistic activity. On the other hand, PL1 cannot grow in PDB liquid medium, but PL1 sub-cultured to PDA solid medium can produce active compounds against Cg. I observed that the cut-agar extraction by EA had the best extraction efficiency. In addition, 1 mg/ mL of EA condensed extracts is the minimum inhibitory concentration on spores-germination-plates. To identify the active compounds produced by PL1, we employed several strategies, including LC-ESI-MS, MALDI-TOF-IMS, MALDI-TOF-MS, HPLC, HRMS, whole genome sequencing (done by Drs. Feng-Chia Hsieh and Chih-Cheng Chien) and transcriptome (done by Dr. Chih-Cheng Chien). After high-performance-liquid-chromatography (HPLC) separation, I observed the 17th fraction had anti-Cg function under our conditions. Then, I used high resolution mass chromatography to identify the active compounds. The active compounds were predicted to be members of

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the syrbactins family, including cepafungin I (*m/z* 535), glidobactin A (*m/z* 521) and glidobactin C (*m/z* 549) (done by Dr. Yu-Liang Yang). Besides, the MS spectra data confirmed the active compounds were highly extracted by agar incubation and the PDA acted as a stress to induce syrbactin compounds. However, the syrbactin signals were not detected by MALDI-TOF MS. It is possible that each compounds had its own characteristics and had different sensitivity to be detected in mass spectrometry. I also assumed that there still had other active compounds in the EA condensed extracts of PL1 which had not been found in our studies (Figure S1).

In summary, I found that syrbactins were induced by the agar incubation. However, whether these compounds can be used as biocontrol agents requires further studies.

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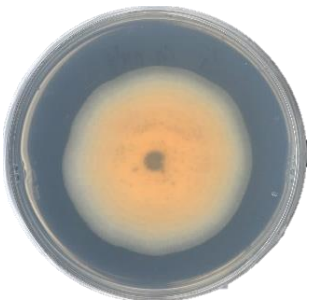
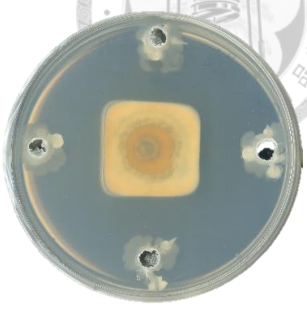
Figures and Tables



CK

***Pseudomonas taiwanensis* sp. nov. CMS^{T} (Pt)**



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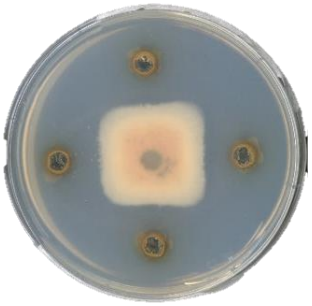

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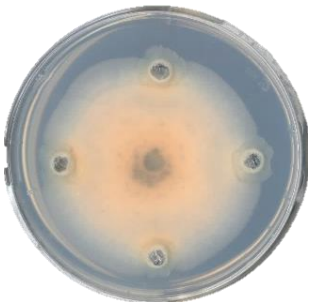

***Photorhabdus akhurstii* sp. nov. 0813-124 phase II (PL2)**

Figure 1. Screening of soil microbes for bacteria with antagonistic activities against *Colletotrichum gloeosporioides* (Cg) by antagonistic assays. Each antagonistic bacterium was screened on PDA plates for 7 days. ASB111 and PL1 had anti-Cg ability on PDA after 7 days antagonistic assay.

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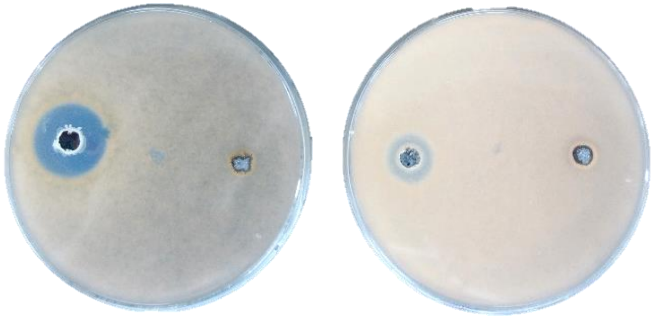

30mg/ mL
1-butanol extracts

30mg/ mL
EA extracts

Figure 2-1. Comparison of the inhibition of germination of Cg spores by the active compounds of ASB111 extracted with different polarity organic solvents. The extracts were tested with 30 mg/mL. 1-butanol extracts of ASB111 was stronger than EA extracts on 2-days-spores-germinated plates.

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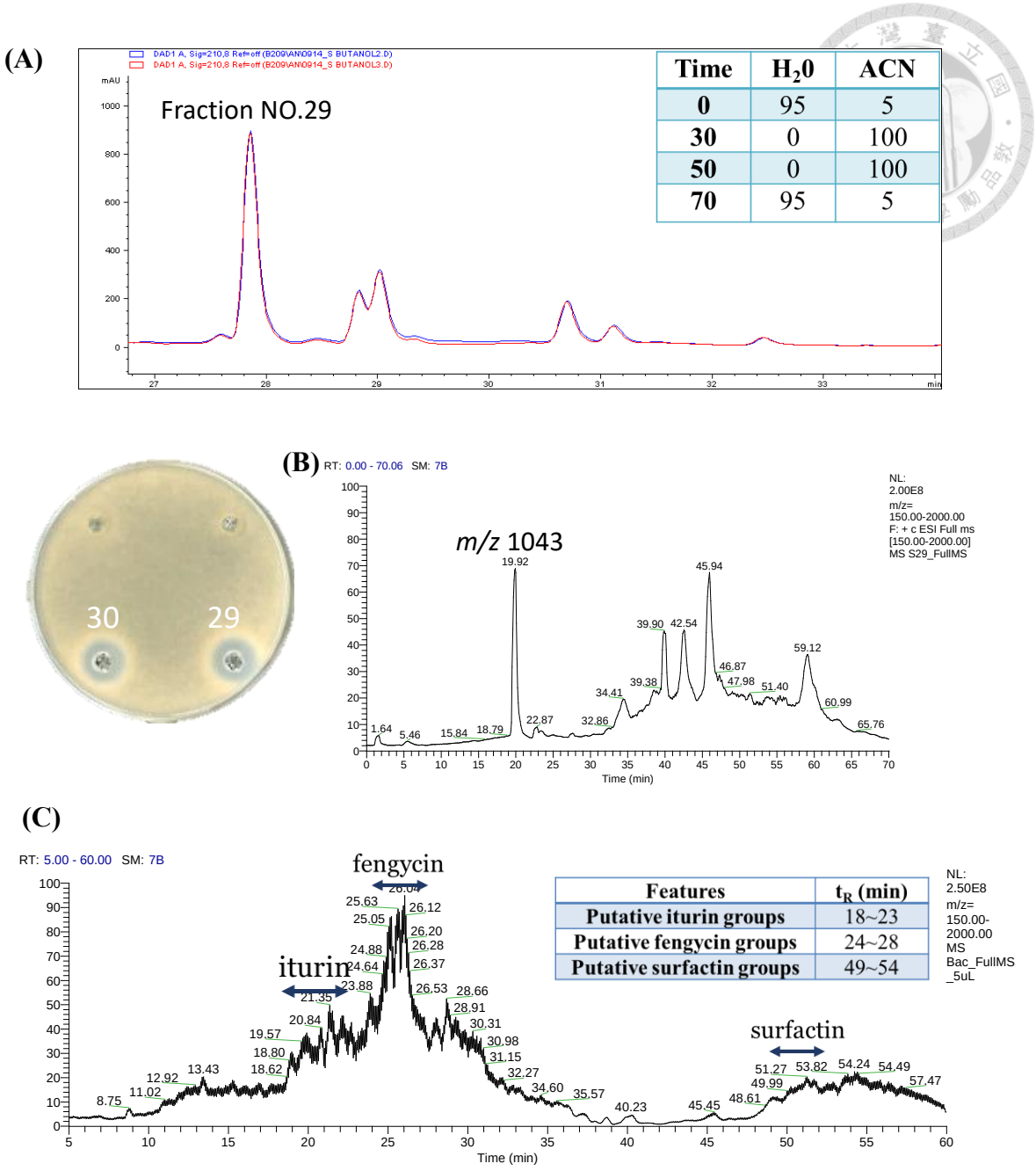

Figure 2-2. Analysis of active compounds produced by ASB111. (A) 1-butanol extracts were separated by C18 RP-HPLC under gradient condition, the 29th fraction had anti-Cg ability on 2-days-spores-germinated plates. **(B)** The 29th fraction was analyzed by LC-

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MS (LCQ), the strongest signal was showed at RT:19 and MS^1 spectra indicated RT:19 was predicted to iturin (*m/z* 1043). **(C)** 1-butanol extracts were analyzed by LC-MS (LCQ) and the active compounds of MS^1 spectra were expected to iturin, fengycin and surfactin.

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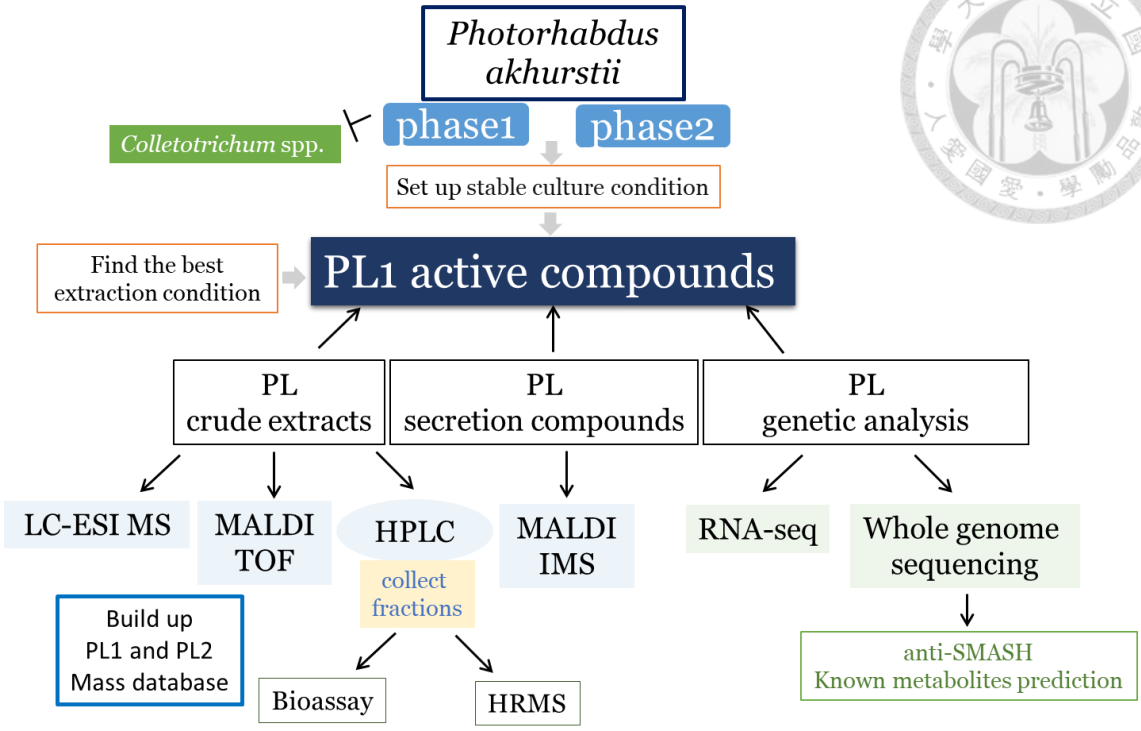

Figure 3. Flow chart of analysis of active compounds produced by *Photorhabdus akhurstii* sp. nov. 0813-124 phase I (PL1).

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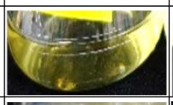
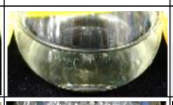
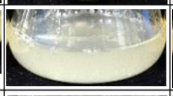
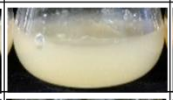
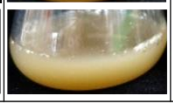
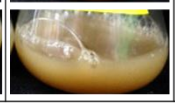
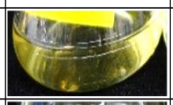
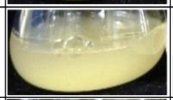
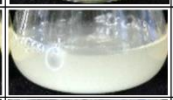
(A)

Culture Time	LB	LP	NB	PP3T	TSB	PDB
0						
24hr						
48hr						
72hr						
96hr						
120hr						

Phenotypes of PL1 cultures grown continuously for 120 hr.

Culture Time	LB	LP	NB	PP3T	TSB	PDB
0						
24hr						
48hr						
72hr						
96hr						
120hr						

Phenotypes of PL2 cultures grown continuously for 120 hr.

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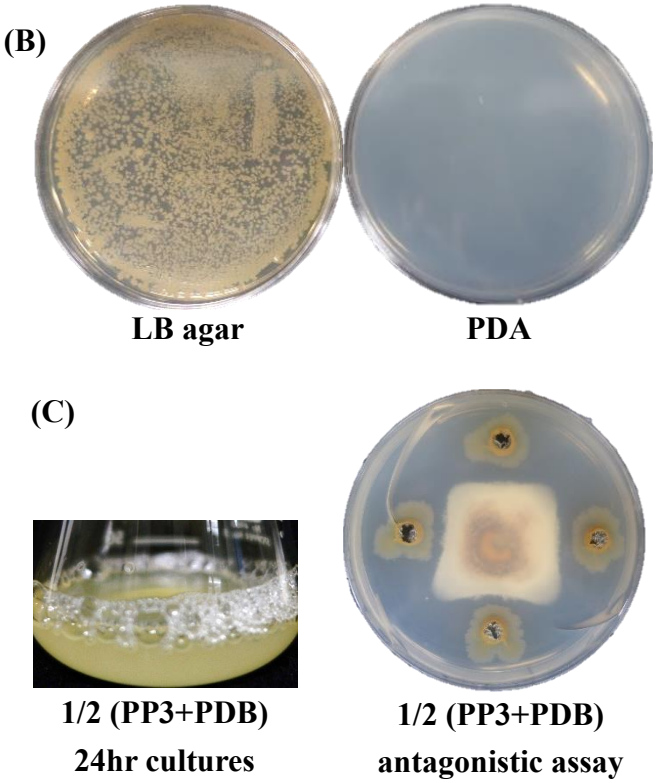

Figure 4-1. Characterization of PL1 and PL2 cultures. (A) PL1 and PL2 were cultured for 120 hrs and the phenotypic variations were recorded from 24 hr to 120 hr with LB, LP, NB, PP3T, TSB and PDB. Each broth was sub-cultured from a single colony of MacConkey agar plate (0.2 cm colony was cultured for 2 days). PL1 cultured broths in LB, PP3T and TSB blacken from 72 hr cultures. PL1 and PL2 could not grow in PDB. **(B)** PL could not grow with the nutrients of PDB by replica plating examination. **(C)** Defined medium: 1/2 (PP3+PDB). 1/2 (PP3+PDB) cultures can have antagonistic activity (Dual culture for 7 days).

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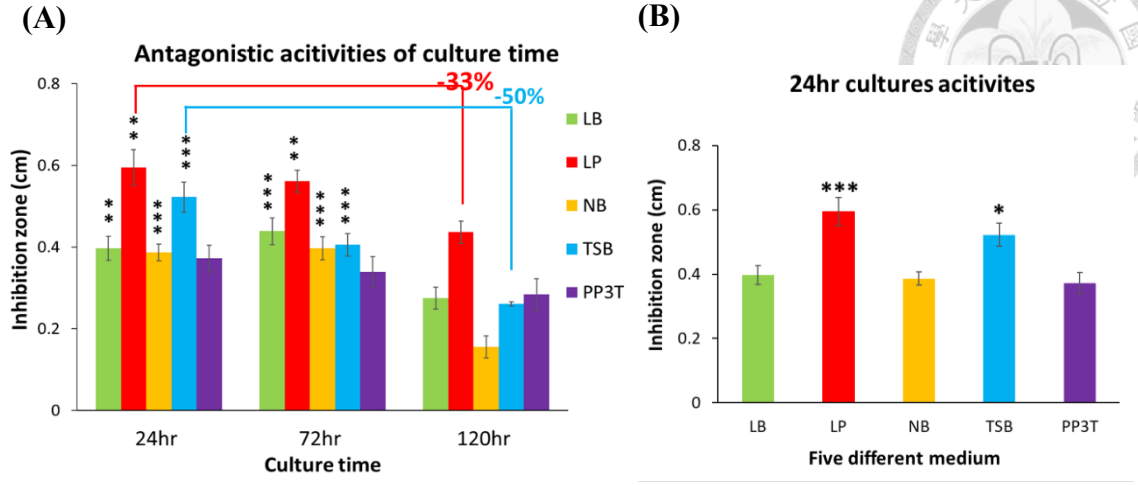

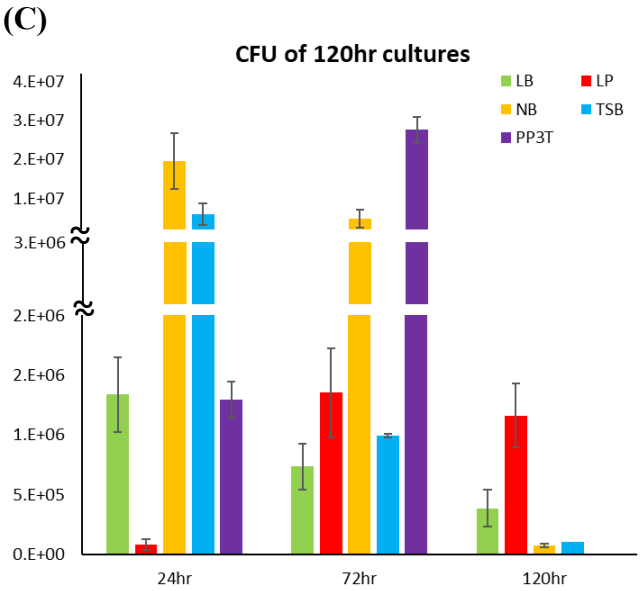

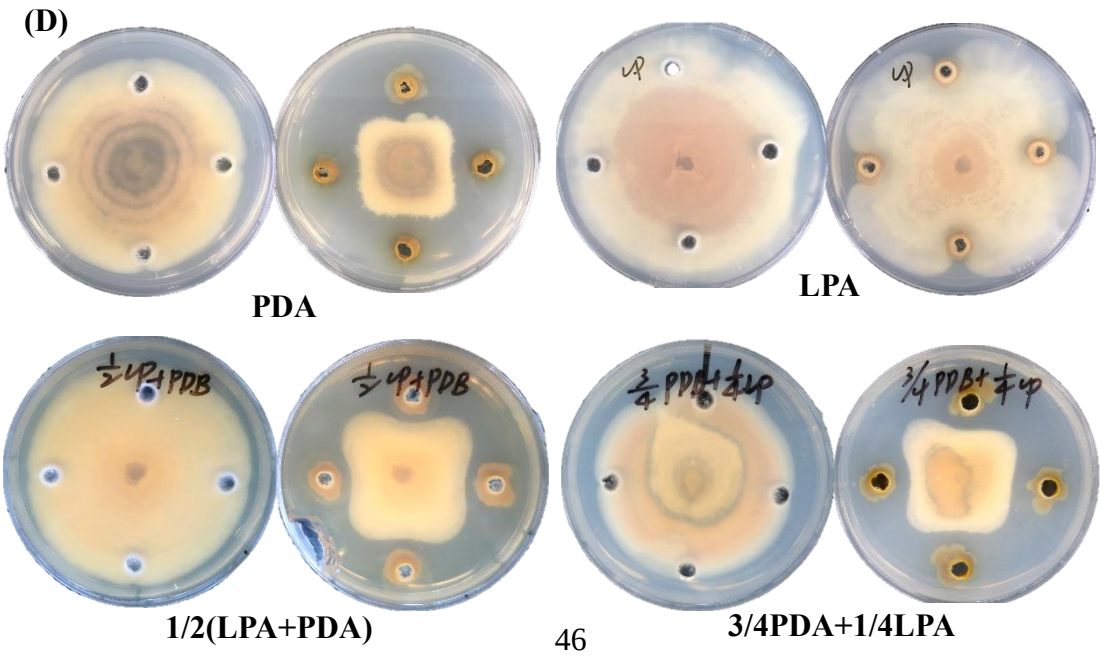

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Figure 4-2. Establishment of PL1 stable culture condition. (A) The antagonistic activities of the 24 hr cultures and the 72 hr cultures were significantly higher than the 120 hr cultures. The antagonistic activity of TSB cultures was declined 50% in 120 hr continuous culture and LP cultures was only declined 33% in 120 hr continuous culture. The results represent the mean ± SE (Four biological replicate). * = P<0.05, ** = P<0.01, *** = P<0.001 **(B)** PL1 showed significantly higher anti-Cg activity when it was grown in LP and TSB medium of 24 hr cultures. The results represent the mean ± SE (Four biological replicate). * = P<0.05, ** = P<0.01, *** = P<0.001 **(C)** NB cultures and TSB cultures showed highest CFU at 24 hr culture time. NB cultures and PP3T cultures showed highest CFU at 72 hr culture time. The CFU of all culture broths were declined. PL1 in LP medium used the less CFU that has the strongest activity at 24 hr culture time. **(D)** LPA, 1/2LPA+1/2PDA and 1/4LPA+3/4PDA cannot have better antagonistic activity of solid medium test. PDA is the best choice to induce antagonistic activity of PL1.

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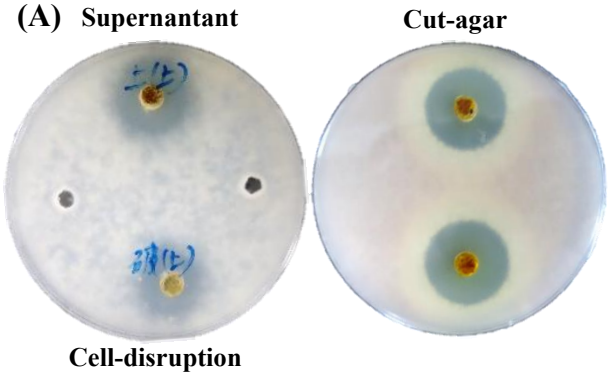

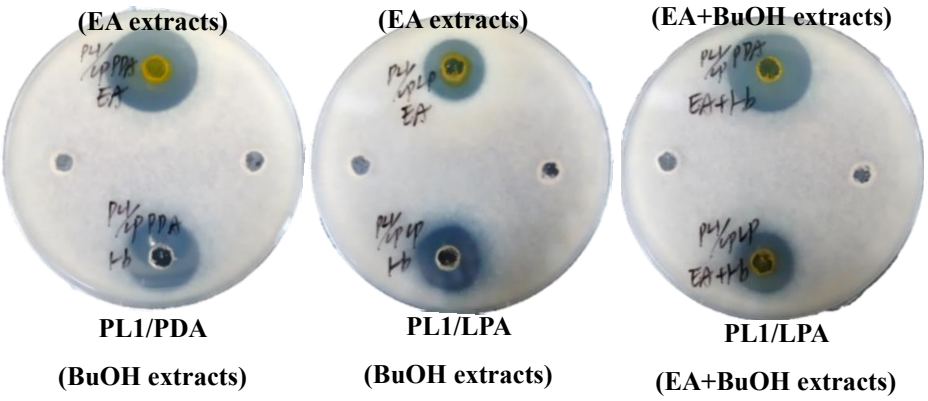

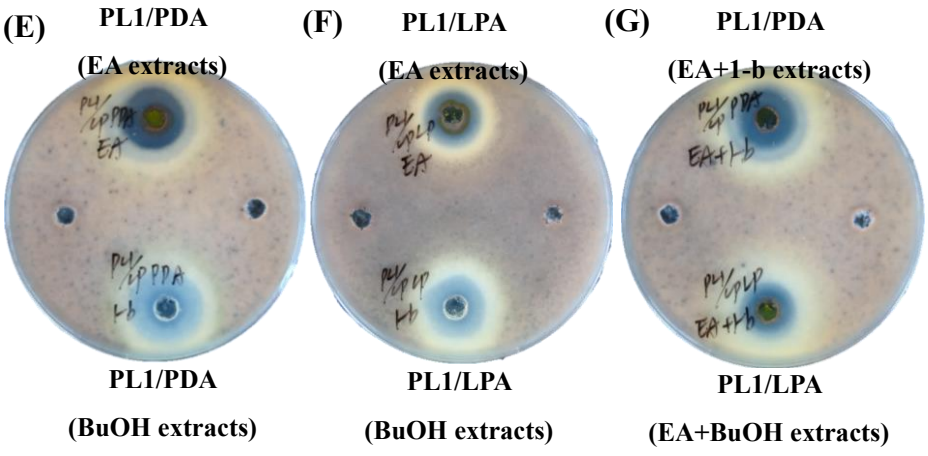

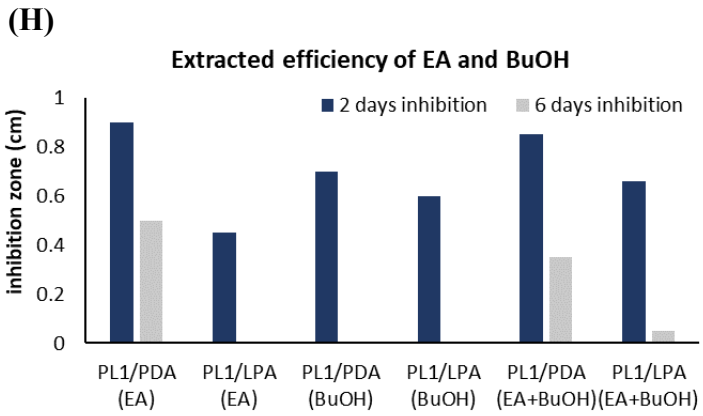

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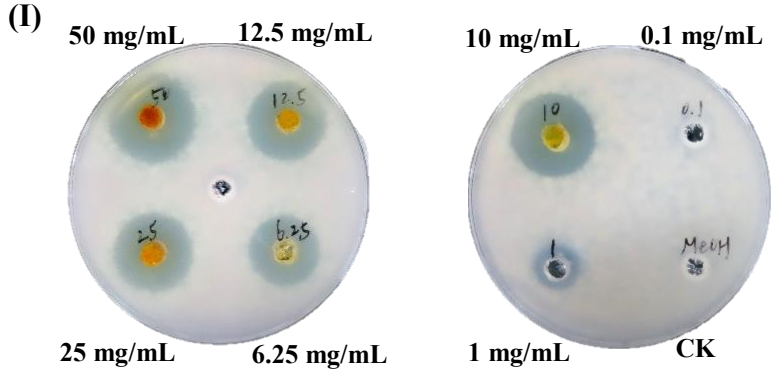

Figure 5. Characterization of active compounds of PL1 extracts. Each experiment was tested on spores-germinated-plate. **(A)** Cut-agar method with EA extracts had better efficiency. **(B)** Comparison of PDA cultures with EA extracts and BuOH extracts (2-days incubated plate). **(C)** Comparison of LPA cultures with EA extracts and BuOH extracts of PL1 (2-days incubated plate). **(D)** Comparison of 1/2EA extracts plus 1/2BuOH extracts with PDA cultures and LPA cultures of PL1 (2-days-spores-germinated-plates). **(E)** Comparison of PDA cultures with EA extracts and BuOH extracts (6-days-spores-germinated-plates). **(F)** Comparison of LPA cultures with EA extracts and BuOH extracts of PL1 (6-days-spores-germinated-plates). **(G)** Comparison of 1/2EA extracts plus 1/2BuOH extracts with PDA cultures and LPA cultures of PL1 (6 day-spores-germinated-plates). EA is the best solvent to extract active compounds of PL1. **(H)** Extracted efficiency of EA and BuOH summary graph. EA extracts had the highest efficiency. **(I)** Serial dilution of EA extracts from 50 mg/ mL to 0.1 mg/ mL. The concentration under 1 mg/ mL gradually lost anti-Cg activity.

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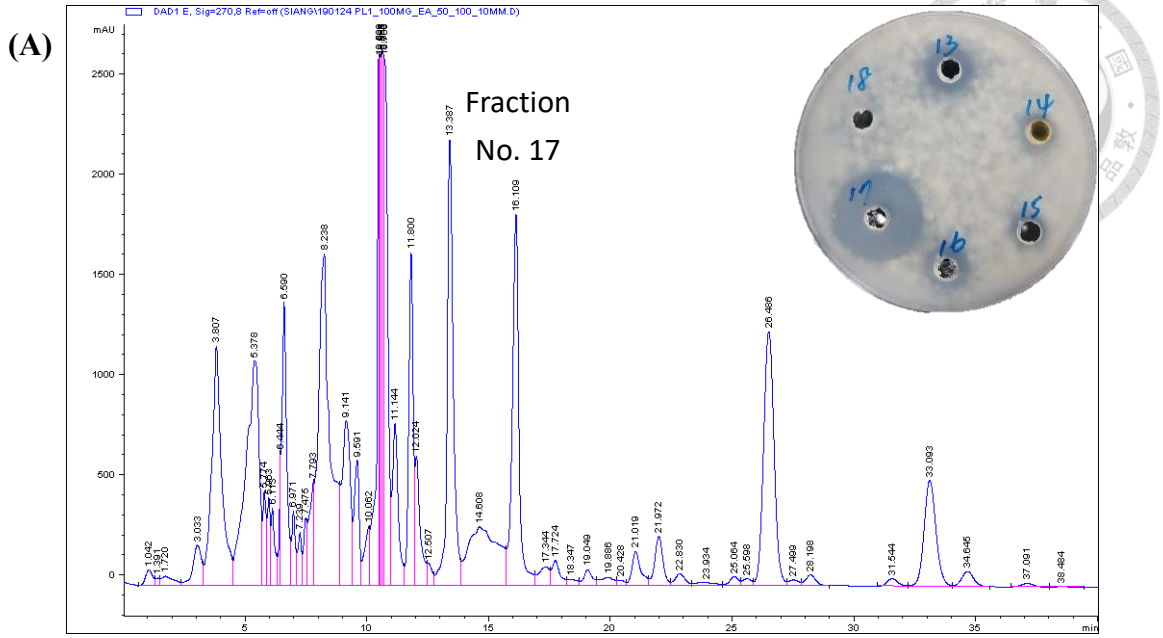

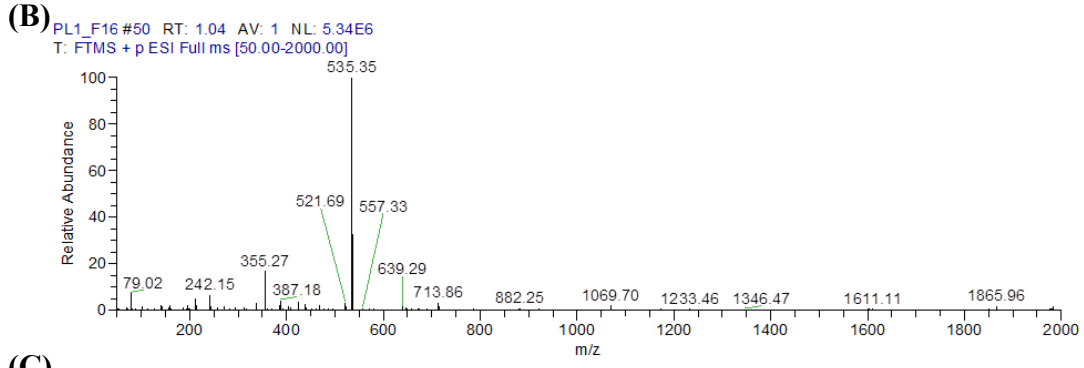

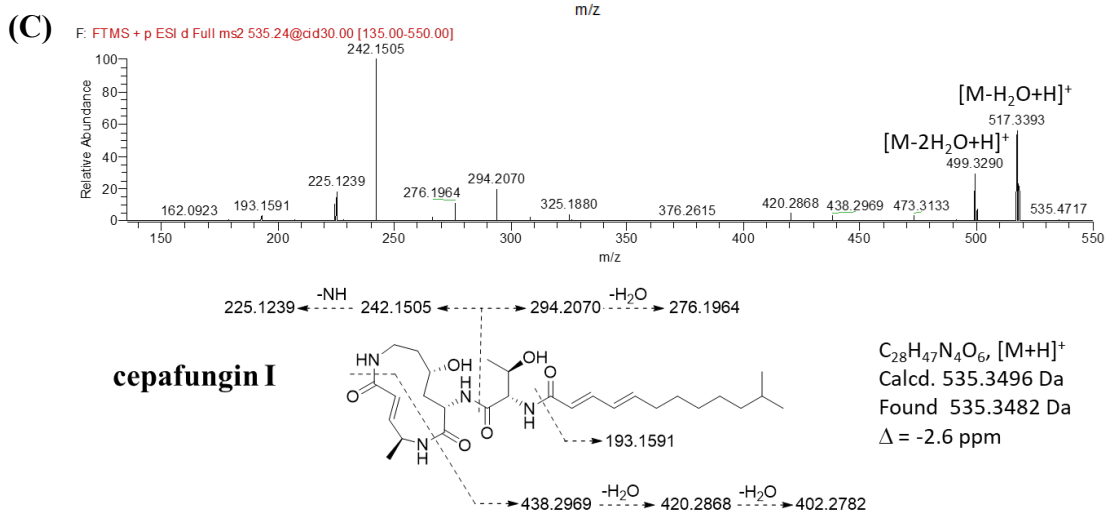

cepafungin I

$C_{28}H_{47}N_4O_6$, $[M+H]^+$
Calcd. 535.3496 Da
Found 535.3482 Da
Δ = -2.6 ppm

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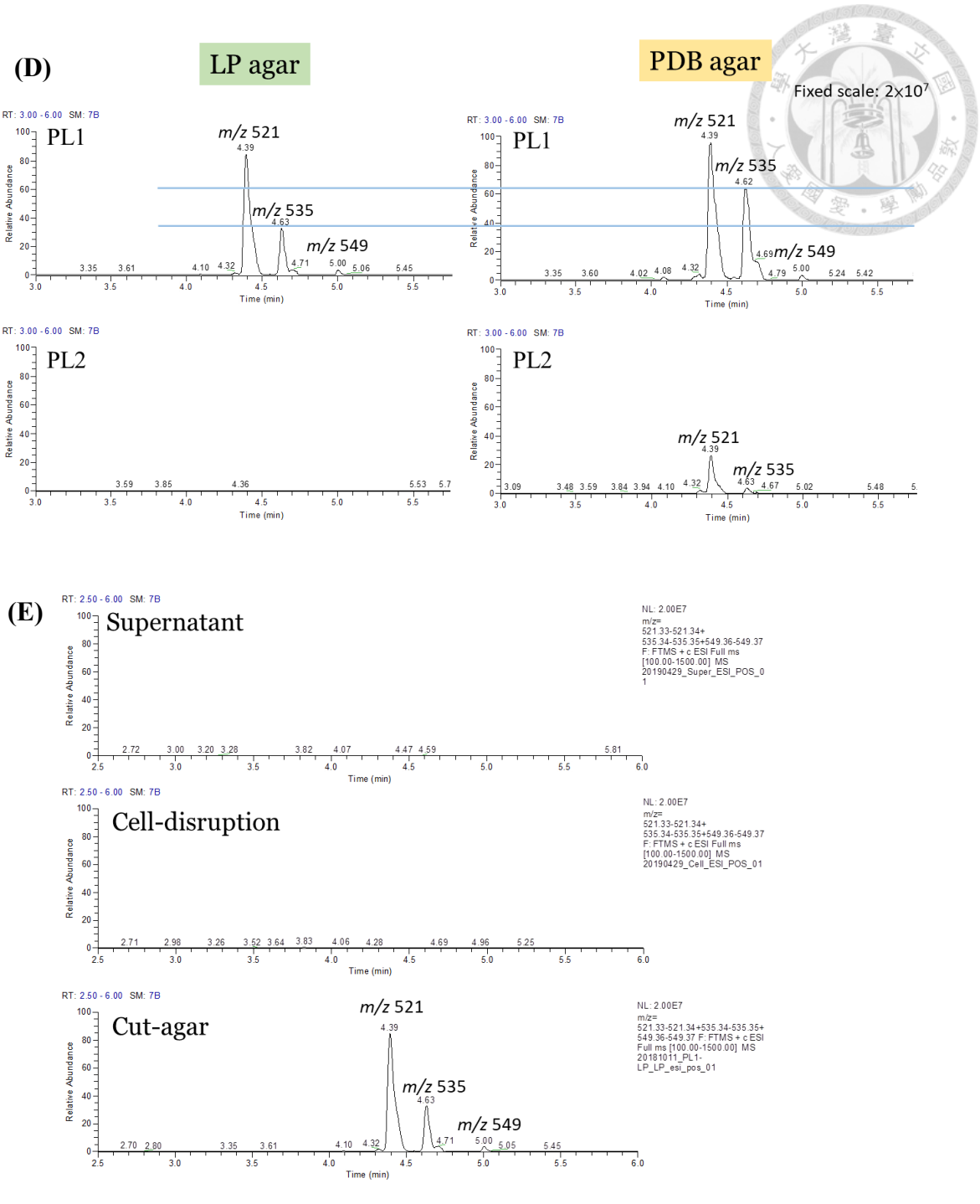

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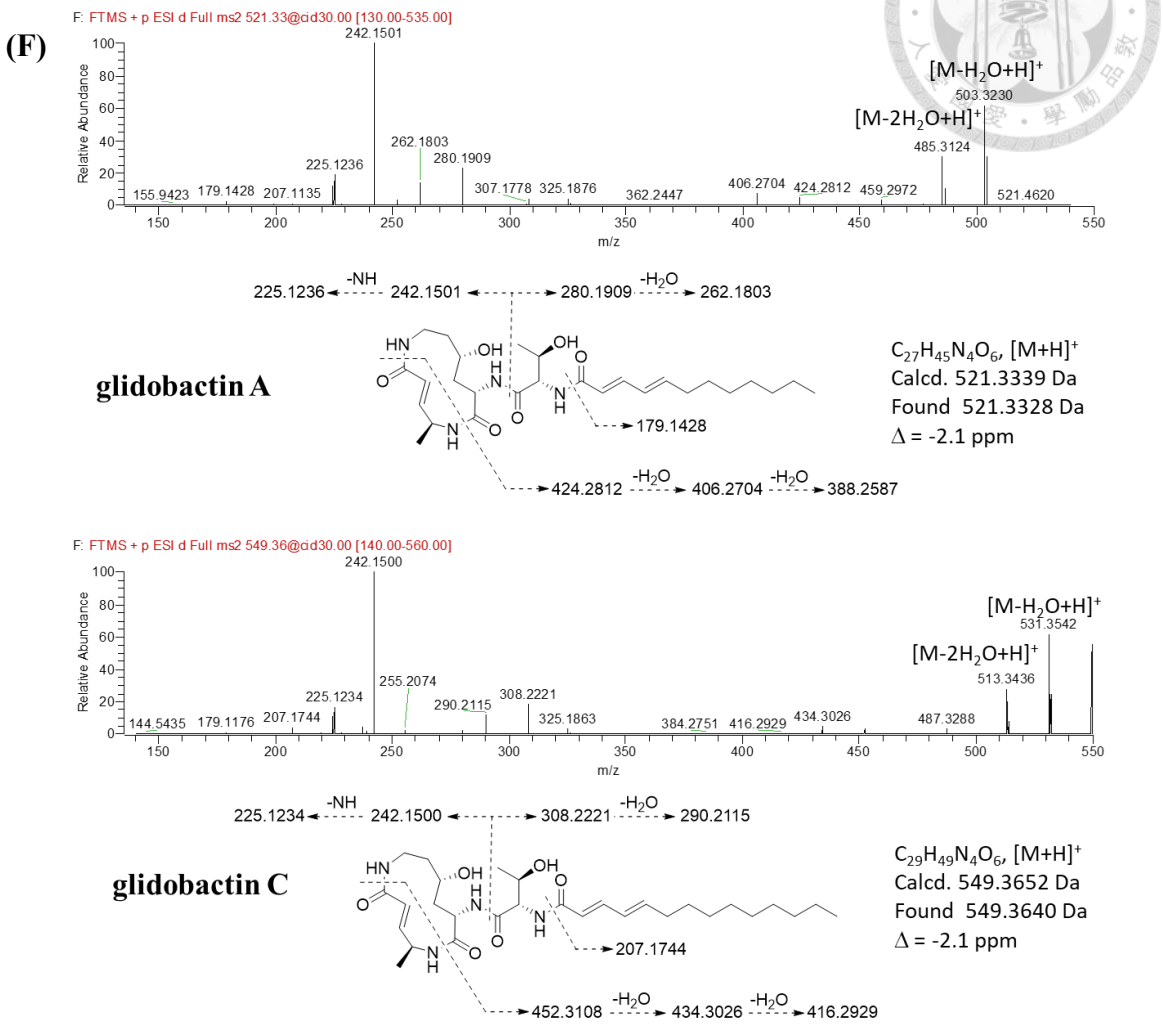

Figure 6. Analysis of active compounds produced by PL1. (A) EA extracts were separated by C18 RP-HPLC under isocratic condition, the 17th fraction had anti-Cg ability on 2-days-spores-germinated plates. **(B)** The 17th fraction was analyzed by high resolution mass spectrometry (HRMS), the MS^1 spectra indicated the active signal was *m/z* 535.35. **(C)** The MS^2 spectra was identified by tandem mass and the active compounds of PL1 was cepafungin I (*m/z* 535). **(D)** PL1 has more cepafungin I of PDA

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cultures (syrbactins compounds; *m/z* 521 glidobactin A, *m/z* 535 cepafungin I, *m/z* 549 glidobactin C). **(E)** Syrbactins family compounds was highly induced with cut agar extraction. **(F)** The other compounds of syrbactins family, glidobactin A (*m/z* 521) and glidobactin C (*m/z* 549), MS^2 spectra were identified by tandem mass (The structures of these compounds were drawn and predicted by Dr. Yu-Liang Yang).

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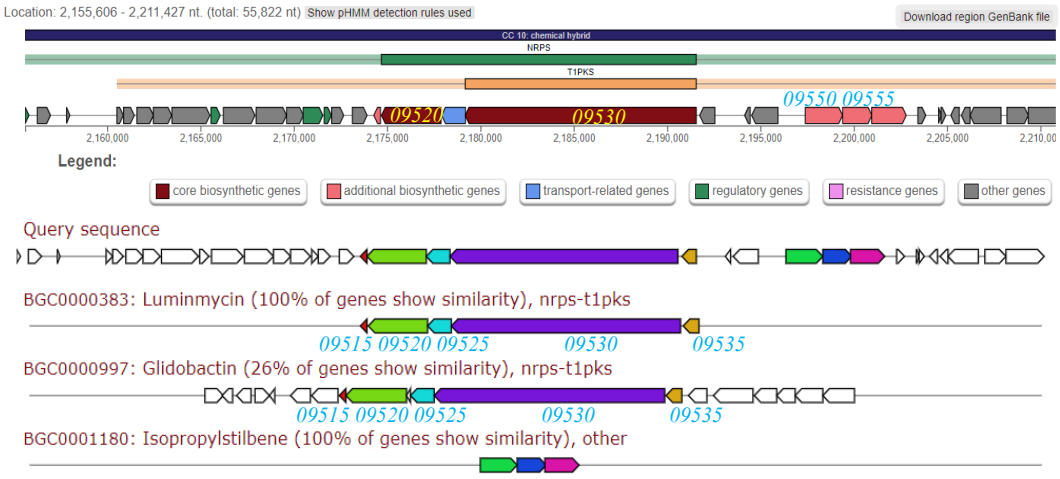
(A)

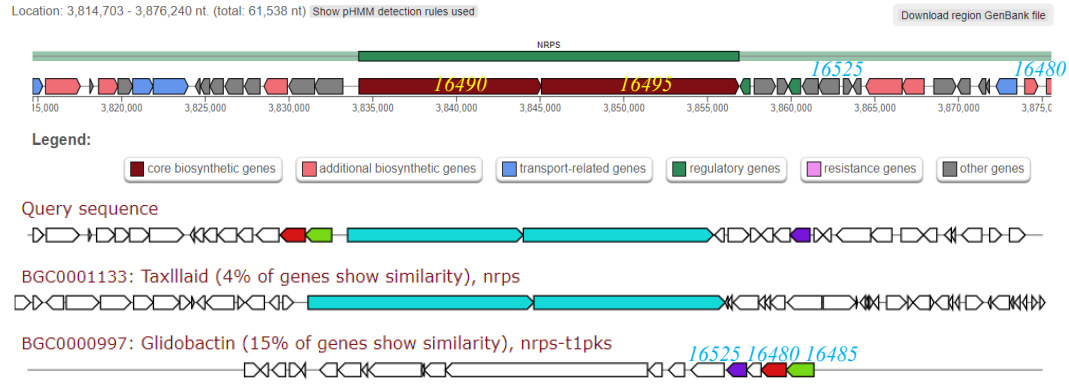
Region	Type	PL1 position		PL2 position		Most similar metabolites		similarity
1	bacteriocin	69,828	80,739	69,824	80,735			
2	PpyS-KS	188,768	209,817	188,761	209,810			
3	CDPS	295,887	328,658	295,897	328,668			
4	terpene	590,148	613,720	590,120	613,692	Carotenoid	terpene	83%
5	NRPS	1,075,673	1,127,820	1,075,634	1,127,781	Xenortide	NRPS	100%
6	NRPS	1,383,998	1,441,333	1,383,950	1,441,284			
7	thiopeptide	1,898,419	1,924,511	1,898,363	1,924,454	O-antigen	saccharide	14%
8	NRPS , T1PKS	2,155,606	2,211,427	2,155,545	2,211,364	Luminmycin	nrps-t1pks	100%
9	arylpolyene	2,404,949	2,446,133	2,404,883	2,446,067			
10	resorcinol	2,514,529	2,555,677	2,514,461	2,555,609	Isopropylstilbene	other	100%
11	NRPS , T1PKS	2,666,544	2,742,482	2,644,779	2,720,716	Yersiniabactin	nrps-t1pks	4%
12	NRPS	3,077,784	3,166,381	3,056,013	3,144,608	Colicin V	ripp	2%
13	NRPS	3,182,648	3,232,086	3,160,872	3,210,310	Turnerbactin	NRPS	23%
14	NRPS	3,621,812	3,780,208	3,600,017	3,758,409	Arthrofactin	NRPS	50%
15	NRPS	3,814,703	3,876,240	3,792,902	3,854,438	Taxlllaid	NRPS	4%
16	NRPS	3,952,008	4,007,276	3,930,202	3,985,469	Luminmide	NRPS	100%
17	T1PKS	4,175,394	4,220,165	4,153,583	4,198,354			
18	betalactone	4,299,828	4,325,327	4,278,017	4,303,515			
19	NRPS	4,523,574	4,568,845	4,501,757	4,547,025	Distamycin	NRPS	14%
20	NRPS , T1PKS	4,855,143	4,910,785	4,833,311	4,888,953			
21	T1PKS , NRPS	4,944,344	5,019,873	4,922,511	4,998,040	Xenocoumacin	nrps-t1pks	78%
22	siderophore	5,410,609	5,435,090	5,388,773	5,413,254	Desferrioxamine E	other	75%

0 10

(B) Region 8



(C) Region 15



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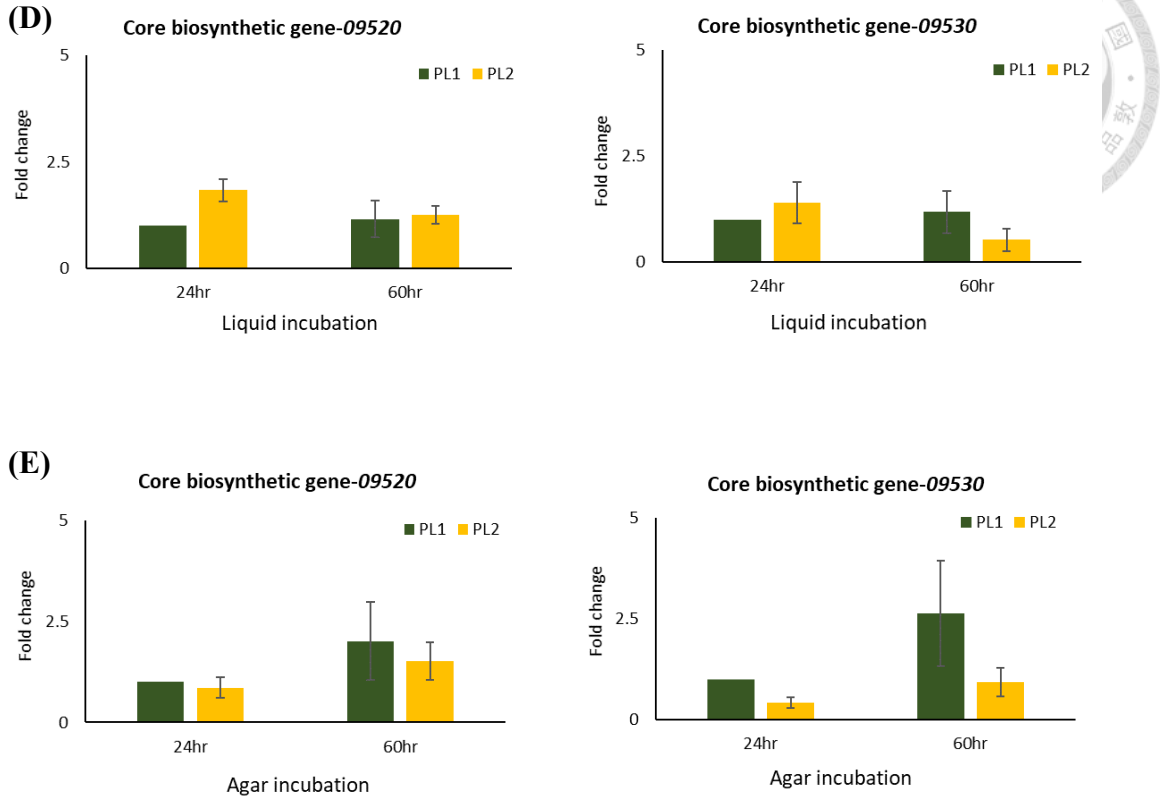

Figure 7. PL genetic analysis. (A) Combination of whole genome sequence and transcriptomic profile. PL1 and PL2 have 22 similar biosynthetic gene clusters (BGCs). Based on transcriptomic heat map analysis, there are 3 gene clusters have different gene expression including region8, 10 and 21 (24 hr cultures RNA extraction, whole genome sequence was done by Dr. Feng-Chia Hsieh and transcriptomic profile was done by Dr. Chih-Cheng Chien). **(B)** The biosynthetic genes analysis of region 8. 26% of genes show similarity to glidobactin in region 8. **(C)** The biosynthetic genes analysis of region 15. 15% of genes show similarity to glidobactin in region 15. **(D)** The expression of core biosynthetic gene, *09520* and *09530* (LP liquid cultures). The fold change is normalized

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to PL1 24hr cultures. **(E)** The expression of core biosynthetic gene, *09520* and *09530* (LP agar cultures). The fold change is normalized to PL1 24hr cultures.

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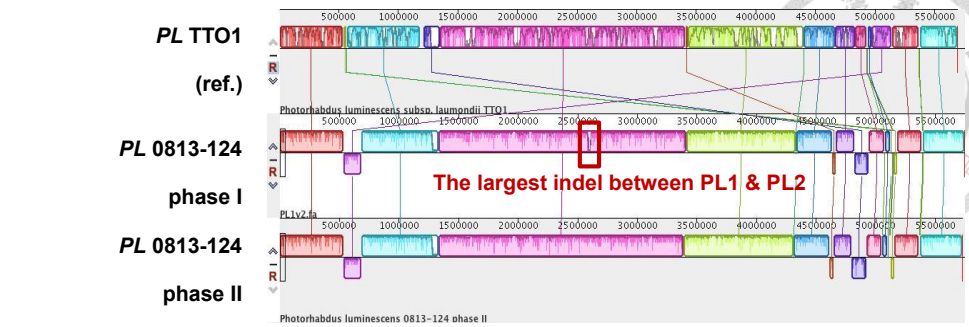
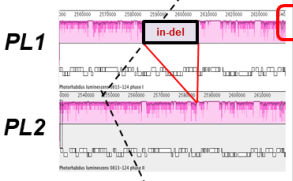
(A)

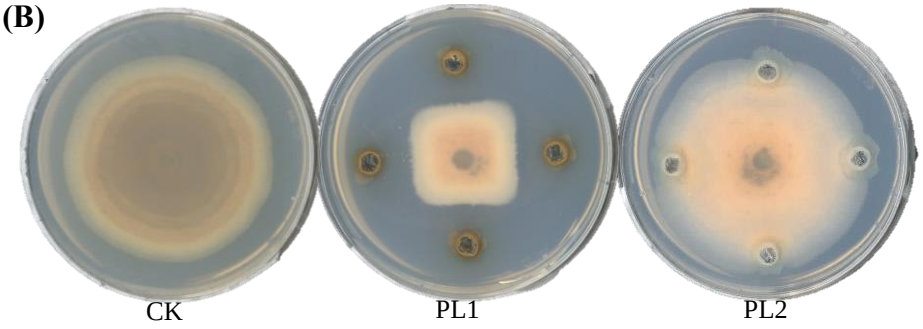


In-del region

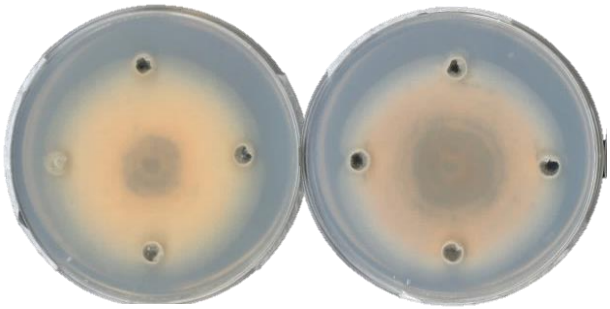


Feature ID	PL1 Expression values	PL1 total gene reads	PL1 RPKM	PL2 Expression values	PL2 total gene reads	PL2 RPKM	Annotation
CE143_11420	231	231	31.66	0	0	0	NADP-dependent 3-hydroxy aciddehydrogenase
CE143_11425	250	250	16.07	0	0	0	histidine ammonia-lyase
CE143_11430	3596	3596	218.09	0	0	0	chitinase
CE143_11435	331	331	27.46	0	0	0	hypothetical protein
CE143_11440	155	155	17.64	0	0	0	Insecticidal toxin
CE143_11445	311	311	33.72	0	0	0	hypothetical protein
CE143_11450	915	915	122.00	0	0	0	MBL fold metallo-hydrolase
CE143_11455	81	81	11.42	0	0	0	hypothetical protein
CE143_11460	529	529	55.94	0	0	0	peptidase S58
CE143_11465	10	10	0.78	0	0	0	FAD/NAD(P)-binding oxidoreductase
CE143_11470	3	3	1.20	0	0	0	(2Fe-2S)-binding protein
CE143_11475	15	15	1.36	0	0	0	D-amino-acid oxidase
CE143_11480	47	47	5.08	0	0	0	hydroxyproline-2-epimerase
CE143_11485	11	11	1.35	0	0	0	AraC family transcriptional regulator
CE143_11490	28	28	3.12	0	0	0	dihydrodipicolinate synthase family protein
CE143_11495	50	50	3.25	0	0	0	aldehyde dehydrogenase (NADP(+))
CE143_11500	155	155	9.94	0	0	0	aspartate:proton symporter

(B)



CK PL1 PL2



CE143_11430 Transformants-1

CE143_11430 Transformants-2

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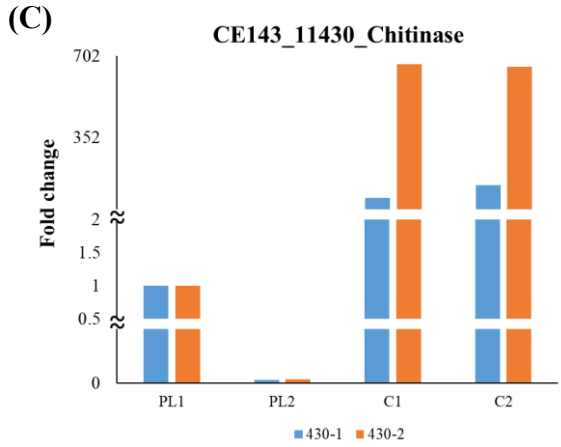

Figure 8. Comparison of PL1 and PL2 genomic structure. (A) Genome structure of PL1 and PL2. A large indel (20 genes) existed between PL1 and PL2 genomes. In the transcriptome of PL1, *CE143_11430* has the highest gene reads among the genes in the indel. **(B)** Antagonistic assays of *CE143_11430* transformants. **(C)** The expression of *CE143_11430*. The fold change is normalized to PL1.

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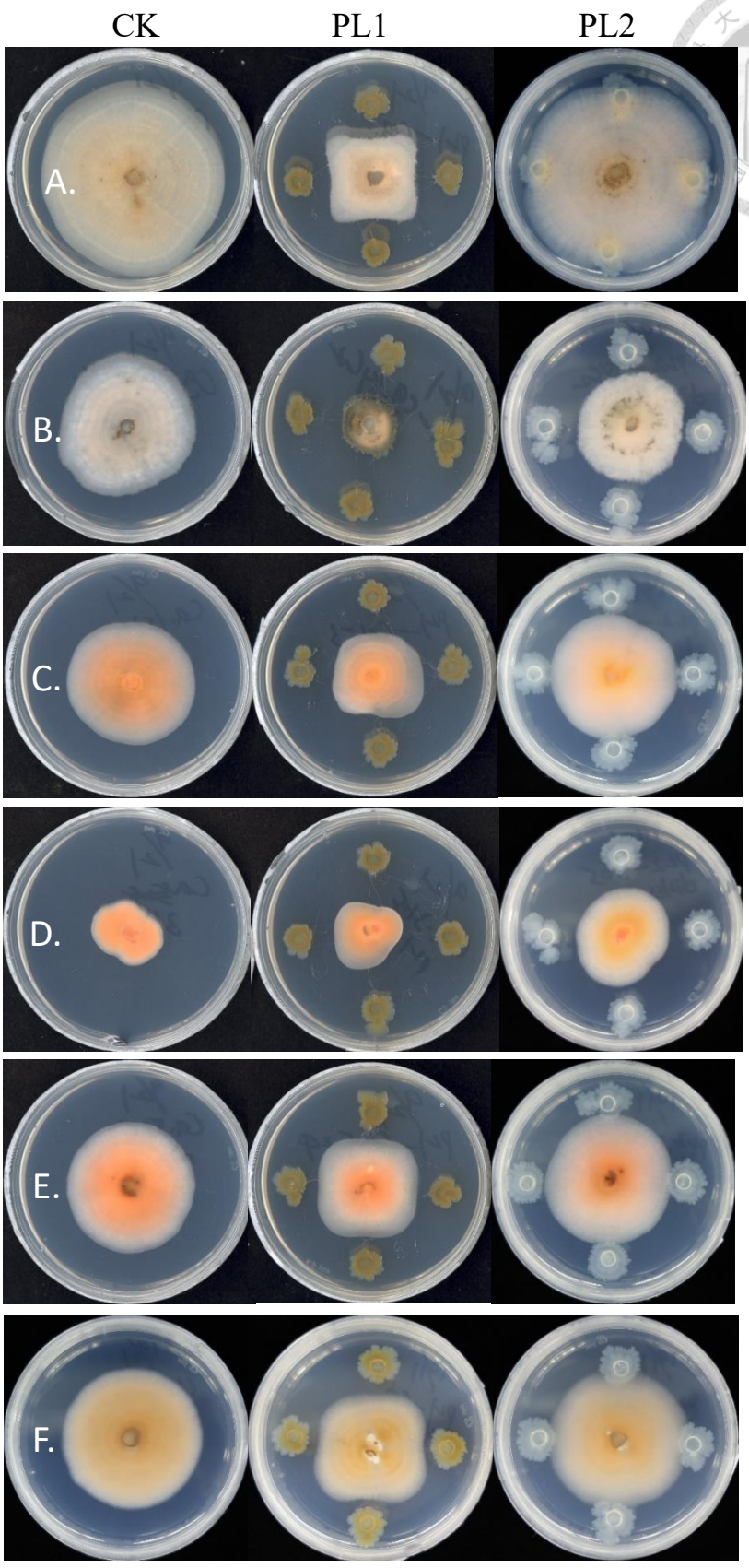

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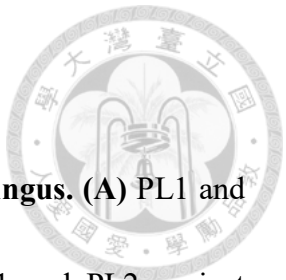

Figure 9. PL1 inhibits a large spectrum of *Colletotrichum* spp. fungus. (A) PL1 and PL2 against *Colletotrichum gloeosporioides* (Strawberry). **(B)** PL1 and PL2 against *Colletotrichum gloeosporioides* Tyc2 (Mango). **(C)** PL1 and PL2 against *Colletotrichum acutatum* 153 (Pepper). **(D)** PL1 and PL2 against *Colletotrichum acutatum* 365 (Pepper). **(E)** PL1 and PL2 against *Colletotrichum acutatum* 524 (Pepper). **(F)** PL1 and PL2 against *Colletotrichum higginsianum* Pa01 (Pai-tsai).

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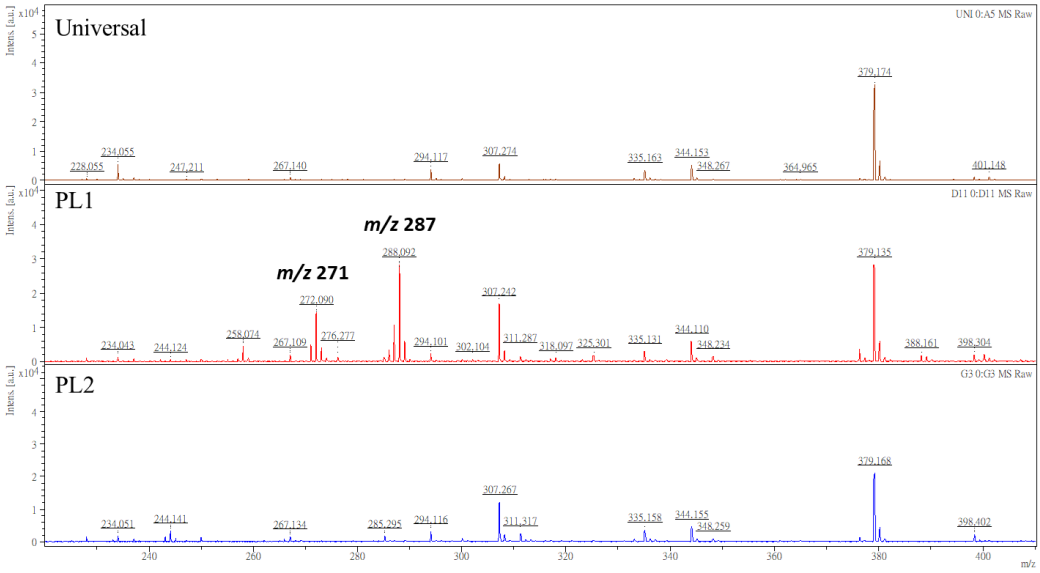
(A)

MALDI TOF	LC-ESI-MS	MALDI IMS
The signals were only detected in PL1		Secreted compounds
m/z: 271	m/z: 255	m/z: 228
m/z: 287	m/z: 271	m/z: 233
	m/z: 287	m/z: 249
		m/z: 265

(B)



(C)

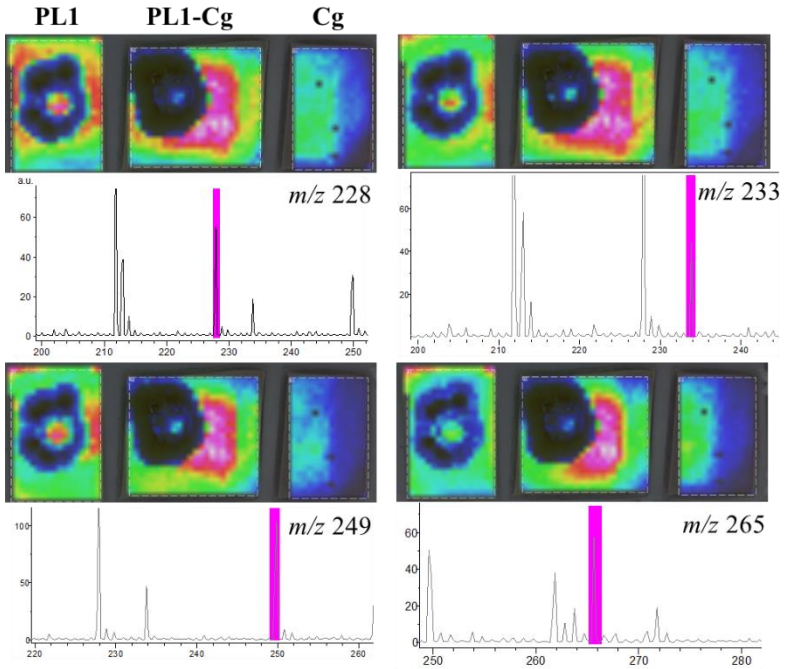


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Figure S1. Analysis of other active compounds in PL1. (A) Other putative compounds produced by PL1 based on LC-ESI-MS, MALDI-TOF-MS and MALDI-TOF-IMS. **(B)** MALDI-TOF-MS analysis of PL1 and PL2. **(C)** MALDI-TOF-IMS analysis of PL1 secreted compounds.

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Table1. List of plant pathogens tested in this study

Plant pathogens	*Strain*	*Host*	*Source*
Colletotrichum gloeosporioides	Non-classified	Strawberry	TACTRI[1]
Colletotrichum acutatum	153	Pepper	PP, NCHU[2]
Colletotrichum acutatum	365	Pepper	PP, NCHU[2]
Colletotrichum acutatum	524	Pepper	PP, NCHU[2]
Colletotrichum higginsianum	PA01	Pai-tsai	PP, NCHU[2]
Colletotrichum gloeosporioides	Tyc2	Mango	PP, NCHU[2]

1: TACTRI, Taiwan Agricultural Chemicals and Toxic Substances Research Institute

2: PP, NCHU, Department of Plant Pathology, National Chung Hsing University

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Table2. List of antagonistic bacteria tested in this study

Antagonistic bacteria	*Strain*	*Host*	*Source*
Pseudomonas taiwanensis	sp. nov. CMS^T	Northern Taiwan soli	IOB, NTU[1]
Bacillus amyloliquefaciens	isolated strain ASB111	Experimental field soil	Our lab
Photorhabdus akhurstii	sp. nov. 0813-124 phase I	Heterorhabditidae brevicaudis	TACTRI[2]
Photorhabdus akhurstii	sp. nov. 0813-124 phase II	Heterorhabditidae brevicaudis	TACTRI[2]

1: IOB, NTU, Institute of Biotechnology, National Taiwan University

2: TACTRI, Taiwan Agricultural Chemicals and Toxic Substances Research Institute

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Table3. Culture media ingredients

Culture media	Ingredients/1L
LB	Tryptone:10g, Yeast extract: 5g, Sodium Chloride: 10g
LP	$Na_2HPO_4 \cdot 7H_2O$: 11.328g, KH_2PO_4: 3g, NaCl: 0.5g, NH_4Cl: 1g, 1% casamino acid: 10g, $MgSO_4 \cdot 7H_2O$: 0.246g, 0.5% glycerol: 5 ml
NB	Beef extract: 3g, Peptone: 5g
PP3T	Proteose peptone NO.3: 20g, Tween20: 5ml
TSB	Tryptone: 17g, Soytone: 3g, Glucose(=Dextrose): 2.5g, Sodium Chloride: 5g, Dipotassium Hydrogen Phosphate: 2.5g
PDB	Potato Starch: 4g, Dextrose: 20g
MacConkey agar	Pancreatic Digest of Gelatin: 17g, Peptones (meat and casein): 3g, Lactose: 10g, Bille Salt No.3: 1.5g, Sodium Chloride: 5g, Agar: 13.5g, Neutral Red: 0.03g, Crystal Violet: 1mg

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Table 4. Active compounds produced by *Photorhabdus luminescens*

Active compound	Activities	References
Isopropylstilbene	Nematode development, Antifungal, Antibacterial	(Joyce et al., 2008)
Rhabduscin	The insect enzyme inhibitor	(Crawford et al., 2012)
Glidobactin	Antifungal, Antitumor	(Groll et al., 2008)
	Proteasome inhibitor	(Oka et al., 1988)
Phurealipids	The insects juvenile hormone epoxide hydrolase inhibitor	(Nollmann et al., 2015)
Photopyrones	As signaling molecules	(Brachmann et al., 2013)
Lumizinones	The inhibitor for cysteine protease	(Park and Crawford, 2016)
Rhabdopeptides/ xenortides	An insect-specific virulence factors	(Reimer et al., 2013)

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Table 5. The primers used for Q-PCR

Gene	Forward sequence	Reverse sequence
r430-1	GAATATTAGGCGATGAGGGTGAA	GCAAATAACGGCGCTTGCT
r430-2	TGATACCCCTCGCTCCGTTA	TCCCCCCAGATTCCTTTCTAA
r16s	TGAAGTGAGAATCAGGACAAACGA	GAACAGGCCCGGGATTG
r09520	CATACTGCTTCCGCTCTCTTCA	AAATCCCGGCAAAGGTCAA
r09530	GGCGACTTCGGGCATACA	GTTAGTACCGCTGCCCAATCTC

doi:10.6342/NTU201902164

References

謝奉家。(2010)。具微生物農藥潛力異小桿線蟲共生細菌光桿菌之特性分析。國立中興大學博士論文。

王昭翔。(2008)。光桿菌 0813-124 抗細菌物質之分離與特性分析。私立朝陽科技大學碩士論文。

植物保護學會會刊 **46:** 163-172, 2004 兼具殺蟲與抗菌作用之線蟲共生細菌－光桿菌

李國譚。2011。草莓園夏季休耕與輪作植株管理。台大農業推廣通訊

李昱輝、呂理燊。2004。草莓病害管理。果菜健康管理研討會專集。

李昱輝、呂理燊。1994。台灣草莓炭疽病。植病會刊 **3:** 256-257

Akhurst, R. J. (1982). Antibiotic activity of Xenorhabdus spp., bacteria symbiotically associated with insect pathogenic nematodes of the families Heterorhabditidae and Steinernematidae. Microbiology, **128:** 3061-3065.

Akhurst, R. J. (1980). Morphological and functional dimorphism in Xenorhabdus spp., bacteria symbiotically associated with the insect pathogenic nematodes Neoaplectana and Heterorhabditis. Microbiology, **121:** 303-309.

Boemare, N. E., & Akhurst, R. J. (1988). Biochemical and physiological characterization of colony form variants in *Xenorhabdus* spp.(Enterobacteriaceae). Microbiology, **134:** 751-761.

Boemare, N. E., Akhurst, R. J., & Mourant, R. G. (1993). DNA relatedness between Xenorhabdus spp.(Enterobacteriaceae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer Xenorhabdus luminescens to a new genus, Photorhabdus gen. nov. Int J Syst Evol Microbiol **43:** 249-255.

Brachmann, A.O., Brameyer, S., Kresovic, D., Hitkova, I., Kopp, Y., Manske, C., Schubert, K., Bode, H.B., and Heermann, R. (2013). Pyrones as bacterial signaling molecules. Nat Chem Biol **9:** 573-578.

Butaitė, E., Baumgartner, M., Wyder, S., & Kümmerli, R. (2017). Siderophore cheating and cheating resistance shape competition for iron in soil and freshwater Pseudomonas communities. Nat. Commun **8:** 414.

Bock, C.H., Shapiro-Ilan, D.I., Wedge, D.E., and Cantrell, C.L. (2013). Identification of the antifungal compound, trans-cinnamic acid, produced by Photorhabdus luminescens, a potential biopesticide against pecan scab. Journal of Pest Science **87,** 155-162.

Chen, W.J., Kuo, T.Y., Hsieh, F.C., Chen, P.Y., Wang, C.S., Shih, Y.L., Lai, Y.M., Liu., J.R., Yang, Y.L., and Shih, M.C. (2016). Involvement of type VI secretion system in secretion of iron chelator pyoverdine in Pseudomonas taiwanensis. Sci Rep **6:** 32950.

doi:10.6342/NTU201902164

Clarke, D. J., & Dowds, B. C. (1995). Virulence mechanisms of Photorhabdus sp. strain K122 toward wax moth larvae. J INVERTEBR PATHOL, **66:** 149-155.

Crawford, J.M., Portmann, C., Zhang, X., Roeffaers, M.B., and Clardy, J. (2012). Small molecule perimeter defense in entomopathogenic bacteria. Proc Natl Acad Sci U S A **109:** 10821-10826.

Daborn, P.J., Waterfield, N., Blight, M.A., and Ffrench-Constant, R.H. (2001). Measuring virulence factor expression by the pathogenic bacterium Photorhabdus luminescens in culture and during insect infection. J Bacteriol **183:** 5834-5839.

Dudnik, A., Bigler, L., and Dudler, R. (2013). Heterologous expression of a Photorhabdus luminescens syrbactin-like gene cluster results in production of the potent proteasome inhibitor glidobactin A. Microbiol Res **168:** 73-76.

Dunphy, G. B., & Webster, J. M. (1988). Virulence mechanisms of Heterorhabditis heliothidis and its bacterial associate, Xenorhabdus luminescens, in non-immune larvae of the greater wax moth, Galleria mellonella. Int J Parasitol, **18:** 729-737.

Ehlers, R.-U. (2010). Current and Future Use of Nematodes in Biocontrol: Practice and Commercial Aspects with Regard to Regulatory Policy Issues. Biocontrol Science and Technology **6:** 303-316.

Forst, S., Dowds, B., Boemare, N., & Stackebrandt, E. (1997). Xenorhabdus and Photorhabdus spp.: bugs that kill bugs. Annual Reviews in Microbiology, **51:** 47-72.

Groll, M., Schellenberg, B., Bachmann, A.S., Archer, C.R., Huber, R., Powell, T.K., Lindow, S., Kaiser, M., and Dudler, R. (2008). A plant pathogen virulence factor inhibits the eukaryotic proteasome by a novel mechanism. Nature **452:** 755-758.

Hsieh, F.C., Tzeng, C.Y., Tseng, J.T., Tsai, Y.S., Meng, M., and Kao, S.S. (2009). Isolation and characterization of the native entomopathogenic nematode, Heterorhabditis brevicaudis, and its symbiotic bacteria from Taiwan. Curr Microbiol **58:** 564-570.

Hancock, J. F., Sjulin, T. M., & Lobos, G. A. (2008). Strawberries. In Temperate fruit crop breeding 393-437. Springer, Dordrecht.

Howard, C. M., Maas, J. L., Chandler, C. L., & Albregts, E. A. (1992). Anthracnose of strawberry caused by the Colletotrichum complex in Florida. Plant Dis, (USA).

Jian, W., He, D., Xi, P., and Li, X. (2015). Synthesis and biological evaluation of novel fluorine-containing stilbene derivatives as fungicidal agents against phytopathogenic fungi. J Agric Food Chem **63:** 9963-9969.

Joyce, S.A., Brachmann, A.O., Glazer, I., Lango, L., Schwar, G., Clarke, D.J., and Bode, H.B. (2008). Bacterial biosynthesis of a multipotent stilbene. Angew Chem Int Ed Engl **47:** 1942-1945.

doi:10.6342/NTU201902164

Kim, P. I., Ryu, J., Kim, Y. H., & Chi, Y. T. (2010). Production of biosurfactant lipopeptides Iturin A, fengycin and surfactin A from Bacillus subtilis CMB32 for control of Colletotrichum gloeosporioides. *J Microbiol Biotechnol*, **20:** 138-145.

Krasomil-Osterfel, K. C. (1995). Influence of osmolarity on phase shift in *Photorhabdus luminescens*. Appl. Environ. Microbiol. **61:** 3748-3749.

Krahn, D., Ottmann, C., and Kaiser, M. (2011). The chemistry and biology of syringolins, glidobactins and cepafungins (syrbactins). Nat Prod Rep **28:** 1854-1867.

Kumar, S.N., and Nambisan, B. (2014). Antifungal activity of diketopiperazines and stilbenes against plant pathogenic fungi in vitro. Appl Biochem Biotechnol **172:** 741-754.

Machado, R.A.R., Wuthrich, D., Kuhnert, P., Arce, C.C.M., Thonen, L., Ruiz, C., Zhang, X., Robert, C.A.M., Karimi, J., Kamali, S., Ma, J., Bruggmann, R., and Erb, M. (2018). Whole-genome-based revisit of Photorhabdus phylogeny: proposal for the elevation of most Photorhabdus subspecies to the species level and description of one novel species Photorhabdus bodei sp. nov., and one novel subspecies Photorhabdus laumondii subsp. clarkei subsp. nov. Int J Syst Evol Microbiol **68:** 2664-2681.

Nollmann, F.I., Heinrich, A.K., Brachmann, A.O., Morisseau, C., Mukherjee, K., Casanova-Torres, A.M., Strobl, F., Kleinhans, D., Kinski, S., Schultz, K., Beeton, M.L., Kaiser, M., Chu, Y.Y., Phan Ke, L., Thanwisai, A., Bozhuyuk, K.A., Chantratita, N., Gotz, F., Waterfield, N.R., Vilcinskas, A., Stelzer, E.H., Goodrich-Blair, H., Hammock, B.D., and Bode, H.B. (2015). A Photorhabdus natural product inhibits insect juvenile hormone epoxide hydrolase. Chembiochem **16:** 766-771.

Orozco, R.A., Molnar, I., Bode, H., and Stock, S.P. (2016). Bioprospecting for secondary metabolites in the entomopathogenic bacterium Photorhabdus luminescens subsp. sonorensis. J Invertebr Pathol **141:** 45-52.

Park, H.B., and Crawford, J.M. (2016). Pyrazinone protease inhibitor metabolites from Photorhabdus luminescens. J Antibiot (Tokyo) **69:** 616-621.

Reblova, M., Gams, W., and Seifert, K.A. (2011). Monilochaetes and allied genera of the Glomerellales, and a reconsideration of families in the Microascales. Stud Mycol **68:** 163-191.

Reimer, D., Cowles, K.N., Proschak, A., Nollmann, F.I., Dowling, A.J., Kaiser, M., ffrench-Constant, R., Goodrich-Blair, H., and Bode, H.B. (2013). Rhabdopeptides as insect-specific virulence factors from entomopathogenic bacteria. Chembiochem **14:** 1991-1997.

doi:10.6342/NTU201902164

Sharma, M., and Kulshrestha, S. (2015). Colletotrichum gloeosporioides: An Anthracnose Causing Pathogen of Fruits and Vegetables. Biosciences Biotechnology Research Asia **12:** 1233-1246.

Shi, D., An, R., Zhang, W., Zhang, G., and Yu, Z. (2017). Stilbene Derivatives from Photorhabdus temperata SN259 and Their Antifungal Activities against Phytopathogenic Fungi. J Agric Food Chem **65:** 60-65.

doi:10.6342/NTU201902164