Molecular crosstalk between plant beneficial rhizobacteria: identification of Pseudomonas compounds influencing the growth and antimicrobial potential of Bacillus velezensis

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Microbial Processes and Interactions

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Résumé

Les Bacilli font partie des bactéries les plus prolifiques en terme de diversité de métabolites bioactifs secondaires (spécialisés) (BSM), notamment des agents chélateurs des métaux, des hormones et des composés antimicrobiens. Ceci est particulièrement vrai pour les membres de l'espèce *B. velezensis* vivant dans la rhizosphère et les plantes. Certains isolats appartenant à cette espèce sont parmi les bactéries les plus prometteuses à utiliser comme agents de biocontrôle pour protéger les plantes contre les phytopathogènes et certains des BSM sont clairement impliqués dans cette activité de biocontrôle. Selon certains travaux récents, y compris ceux réalisés dans le laboratoire MiPI, il semblerait que la production de BSM par *Bacillus* puisse subir des modifications imprévues lors des interactions entre espèces (ou règne). Cependant, peu de publications ont rapporté des résultats d'interactions de *Bacillus* avec d'autres espèces bactériennes du sol hautement compétitives et il est donc mal connu si cette bactérie est capable d'établir des conversations moléculaires avec d'autres espèces qui pourraient avoir un impact important sur l'expression de leur métabolome secondaire respectif.

Notre objectif global est de mieux comprendre dans quelle mesure l'expression de cet arsenal BSM peut être modulée lors d'une interaction avec d'autres bactéries de la rhizosphère et quels sont les signaux moléculaires impliqués. Plus spécifiquement, nous avons sélectionné la souche *Pseudomonas sp*. CMR12a en tant que partenaire d'interaction car cette bactérie est également un bon concurrent produisant une gamme de BSM et est bien caractérisée en ce qui concerne son contenu génomique. *B. velezensis GA1* et *Pseudomonas sp. CMR12a* interagissent en fait de manière multiforme: d'une part, la perception de certains métabolites de *Pseudomonas* déclenche la production de PKS antimicrobiennes par *Bacillus* et, d'autre part, *Pseudomonas* est capable d'inhiber la croissance de *Bacillus*.
Dans ce travail, nous démontrons que le lipopeptide cyclique sessiline est la molécule sécrétée par *P. sp.* CMR12a principalement responsable de l'activité antimicrobienne vis-à-vis de *B. velezensis* GA1. Nous montrons également que la toxicité de la sessiline est neutralisée en présence de surfactine, un lipopeptide synthétisé par *Bacillus*, probablement via une liaison chimique conduisant à la co-précipitation des deux molécules. Cela représente un nouveau rôle écologique pour la surfactine agissant comme une sorte de barrière chimique, qui s'ajoute aux multiples stratégies d'évitement de Bacillus.
Dans ce travail, nous démontrons que le lipopeptide cyclique sessiline est la molécule sécrétée par *P. sp.* CMR12a principalement responsable de l'activité antimicrobienne vis-à-vis de *B.velezensis* GA1. Nous montrons également que la toxicité de la sessiline est neutralisée en présence de surfactine, un lipopeptide synthétisé par *Bacillus*, probablement via une liaison chimique conduisant à la co-précipitation des deux molécules. Cela représente un nouveau rôle écologique pour la surfactine agissant comme une sorte de barrière chimique, qui s'ajoute aux multiples stratégies d'évitement de Bacillus.
De plus, nos données suggèrent fortement que la carence en fer induite par les sidérophores de *P. sp. CMR12a* agissent comme un déclencheur perçu par *B. velezensis* GA1 qui, en réponse,

stimule la production de PKS antibactérienne. Ces résultats illustrent non seulement une nouvelle facette des interactions compétitives à médiation sidérophore pouvant survenir entre deux bactéries de la rhizosphère, mais montrent également que *B. velezensis* peut déclencher une réponse agressive lors de la détection de concurrents.
Dans son humble contribution, ce travail met donc en évidence la très grande complexité des interactions entre espèces pouvant survenir dans la rhizosphère. Compte tenu en particulier de *B. velezensis*, ces résultats inattendus ont probablement une incidence non seulement sur son aptitude écologique, mais également sur son potentiel de contrôle biologique

Mots clés: *Bacillus velezensis* GA1, *Pseudomonas* sp. CMR12a, Interaction, communication, BSMs, PGPR

Abstract

Bacilli are among the most prolific bacteria regarding the potential to form a wide array of Bioactive Secondary (specialized) Metabolites (BSMs) including metal chelators, hormones, and antimicrobials. This is particularly true for members of the rhizosphere-dwelling and plant-associated species *B. velezensis*. Some isolates belonging to this species are among the most promising bacteria to be used as biocontrol agents to protect plants against phytopathogens and some of the BSM are clearly involved in this biocontrol activity. According to some recent works including those performed in the MiPI lab, it appears that BSM production by *Bacillus* may undergo unanticipated changes upon interspecies(kingdom) interactions. However few publications reported outcomes from *Bacillus* interactions with other highly competitive soil bacterial species and therefore, it is poorly known whether this bacterium is able to establish molecular cross-talks with other species which could markedly impact expression of their respective secondary metabolome.

Our global objective is to better appreciate how far the expression of this BSM arsenal can be modulated upon interaction with other rhizosphere bacteria and what are the molecular signals involved. More specifically, we selected *Pseudomonas* sp. strain CMR12a as interacting partner because this bacterium is also a good competitor producing a range of BSM and is well characterized regarding its genomic content. *B. velezensis* GA1 and *Pseudomonas* sp. CMR12a actually interact in a multifaceted way as on one hand, the perception of some *Pseudomonas* metabolites triggers the production of antimicrobial PKS by *Bacillus* and on the other hand, *Pseudomonas* is able to inhibit the growth of *Bacillus*.

In this work, we demonstrate that the cyclic lipopeptide sessilin is the molecule secreted by *P.* sp. CMR12a mainly responsible for the antimicrobial activity toward *B.velezensis* GA1. We also illustrate that sessilin toxicity is neutralized in the presence of surfactin, a lipopeptide synthesized by Bacillus, probably via chemical binding leading to co-precipitation of the two molecules. This represents a new ecological role for surfactin acting as a kind of chemical barrier, which add to the multiple avoidance strategies of *Bacillus*.

Moreover, our data strongly suggest that iron deficiency induced by *P.* sp. CMR12a siderophores acts as trigger perceived by *B. velezensis* GA1 which in response, stimulates the production of antibacterial PKS. These results not only illustrate a new facet of siderophore-mediated competitive interactions that may occur between two rhizosphere bacteria but also show that *B. velezensis* can mount an aggressive response upon sensing competitors.

In its humble contribution, this work thus highlights the tremendous complexity of interspecies interactions that may occur in the rhizosphere. Considering *B. velezensis* specifically, these unsuspected outcomes probably impact not only its ecological fitness but also its biocontrol potential.

Keywords: *Bacillus velezensis* GA1, *Pseudomonas* sp. CMR12a, Interaction, Crosstalk, BSMs, PGPR

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Abbreviations	Meanings	Abbreviations	Meanings
aa	Amino acids	N	Nitrogen
ACN	Acetonitrile	NB-LRR	Nucleotide-binding–leucine-rich repeat
AHL	N-acyl homoserine lactone	NO	Nitric oxide
AI	Auto-inducer	NRPS	Nonribosomal peptide synthetases
BSM	Bioactive secondary metabolite	NSI	Nutritional similarity index
C	Carbon	OD	Optical density
CAA	Casamino acids medium	OSMAC	One strain, multiple compounds
CAGR	Compound annual growth rate	P	Phosphorus
CLPs	Cyclic lipopeptides	PAMP	Pathogen-associated molecular pattern
DAD	Diode array detector	PCA	Phenazine-1-carboxylate
DAMP	Damage associated molecular pattern	PCN	Phenazine-1-carboxamide
DIP	2,2′-bipyridyl	PGPB	Plant growth promoting bacteria
ESI, ES	Electrospray ionization	PGPR	Plant growth promoting rhizobacteria
ETI	Effector triggered immunity	PKS	Polyketide synthases
Fe	Iron	PR	Pathogenesis-related (protein)
HLS	Homoserine lactone	PRR	Pattern-recognition receptors
HPLC	High pressure liquid chromatography	PTI	PAMP-triggered immunity
ISR	Immune system resistance	Pv.	Pathovar
K	Potassium	QS	Quorum sensing
KB	King B medium	Q-TOF	quadrupole-Time-of-flight
LB	Luria–Bertani medium	RE	Root exudates medium
LBA	gelified LB medium	ROS	Reactive oxygen species
LC	liquid chromatography	SAR	Systemic acquired resistance
LP	Lipopeptide	subsp	Subspecies
MAMP	Microbe-associated molecular patterns	UPLC	Ultra-performance liquid chromatography
Mn	Manganese	WLIP	White line inducing principle
MS	Mass spectrometry	Wt	wild type

Chapter 1 :Introduction

1. State of the Art

1.1. General context

Since the dawn of agriculture, men have fought insects, weeds and microorganisms to ensure sufficient food production. For centuries, we had to suffer from these pests as we had little to no ways to fight them. Everything changed when, with the advent of science, an extensive use of chemicals was settled. This recourse to pesticides brought us to an age of abundance where hunger is no longer common in our regions (Gerhardson, 2002). However, this golden age of pesticides brought many issues in its wake. These molecules appeared to threaten people's health and the environment, leading to an exacerbated fear in the public eye, often beyond scientific evidence (Gerhardson, 2002). Aside from this distrust, pests have built up numerous resistances to active substances (Gerhardson, 2002) and they still cause a depletion of 25% of crop yield every year (Borriss, 2015). Moreover, the food demand is expected to double by 2050 whereas cultivated surfaces are expected to decrease (Borriss, 2015). The underlying need for alternative solutions has never been as important as this one: to feed the world. Among the alternatives, the biological ones offer solutions that appear to be more effective in some cases, like for soil borne pathogens. Biocontrol, one particular biological alternative, is expected to play a significant role in this complex problem as it is seen as a relevant alternative to conventional pesticides (Borriss, 2015). However, it still needs to be developed and fully understood to reveal its true potential (Gerhardson, 2002).

1.2. Biocontrol and PGPR

A biocontrol agent is defined as a product either derived-from or containing living microorganisms that can prevent or suppress pests like plant pathogens, insects, and weeds. Biocontrol agents can include living microbes (bacteria, fungi, nematodes, viruses and protozoa), bioactive compounds (such as secondary metabolites, or naturally derived material such as plant extracts) (Kiewnick, 2007; Parnell et al., 2016).

The plant growth promoting bacteria (PGPB) are a specific biocontrol agent which, as its name points out, is based on the use of plant-beneficial microbes. They can also be called plant growth promoting rhizobacteria (PGPR) when located on the roots. They act in various ways:

alleviation of plant stress (water depletion, salt stress,…), regulation of plant growth (by the production of plant hormones), increase of nutrient availability for the plant (like P and Fe by means of siderophores), and inactivation or sequestration of environmental pollutants. However, they are mostly well-known for the enhancement of plant resistance toward pathogens they procure (Vacheron et al., 2013; Borriss, 2015).

1.3. Impact of the PGPR on plant protection

1.3.1. Antagonism

An antagonism is a phenomenon where a PGPR produces chemicals that inhibit the growth or kill another organism. Antagonisms are beneficial for a plant when the organisms they affect are some of this plant's pathogens. Interestingly, PGPRs are well-known for their ability to produce a large amount of bioactive secondary metabolites (BSMs), including many antimicrobials (Debode et al., 2007; Glick, 2012).

1.3.2. Competition

Competition is a mechanism where two organisms fight for an ecological niche (i.e. fight for space or nutrients). It is one of the mechanisms involved in the PGPR biocontrol activity (Ellis et al., 1999; Debode et al., 2007). The aggressive colonization of root surfaces by PGPR and the consumption of nearby nutrients tackle the ability of the pathogen to settle on the root and to further infest it (Glick, 2012). For instance, the nutritional similarity index (NSI)[1] for *P. fluorescens*54/96 (a known PGPR) and *P. ultimum* (a plant-pathogen) is 0.859. This high value indicates that both the PGPR and the pathogen seek the same nutrient sources, and thus the same ecological niche. Consequently, there is a strong competition between these two organisms, which is recognized as part of the biocontrol activity of *P. fluorescens* 54/96 (Ellis et al., 1999).

[1] The nutrition similarity index is calculated with the formula : $NSI = \frac{2n_b}{n_{bca}+n_{path}}$ where n_b is the number of carbon sources utilized by both organisms and n_{bca} and n_{path} are the numbers of carbon sources used by the potential biocontrol agent and the pathogen respectively (Ellis et al., 1999).

1.3.3. ISR and plant immunity

- **Plant immunity**

In a very succinct way, the plant immune system works by means of pattern-recognition receptors (PRRs). These receptors are either able to recognize common microbial compounds, called pathogens- or microbe-associated molecular patterns (PAMPs or MAMPs, such as bacterial flagellin or fungal chitin), or respond to endogenous plant-derived signals that arise from damage caused by enemy invasion, (damage-associated molecular patterns, or DAMPs). The receptor then triggers a cascade of reactions leading to the activation of defenses called PAMP-triggered immunity (PTI). PTI includes the production of reactive oxygen species (ROS), reactive nitrogen species such as nitric oxide (NO), antimicrobial compounds, alterations of the plant cell wall, and the synthesis of pathogenesis-related (PR) proteins (Newman et al., 2013; Pieterse et al., 2014).

In response to this immune reaction, many pathogens have developed strategies to minimize the host immune stimulation and bypass the first line of defense by either suppressing PTI signaling or preventing detection by the host. Plants then came up with a second line of defense: NB-LRR (nucleotide-binding-leucine-rich repeat), a type of receptor protein able to detect specific pathogenic effector resulting in effector triggered immunity (ETI), usually leading to apoptosis (Pieterse et al., 2014).

Systemic acquired resistance (SAR), a priming phenomenon[2], is an enhanced defensive capacity of undamaged plant tissues, distal from the pathogen intrusion. This is due to the perception of long-distance signals coming from PTI or ETI triggered tissues (Conrath, 2011; Pieterse et al., 2014).

- **Induced systemic resistance**

PGPRs have the ability to trigger the plant immunity through a mechanism called 'Induced Systemic Resistance' (ISR) (Pieterse et al., 2014). ISR is defined as a phenomenon where the plant is set in a state of resistance by a chemical inducer, also called elicitor. This state leads to an acute resistance of non-exposed plant parts against future attacks by pathogenic microbes and herbivorous insects and helps the plant overcome abiotic stresses. For instance, ISR leads

[2] Priming is the phenomenon that enables cells to respond to very low levels of a stimulus in a more rapid and robust manner than non-primed cells (Conrath, 2011).

to the activation of defense-related enzymes, signaling of plant hormones, etc. (Conrath, 2011; Walters et al., 2013). The elicitor is a non-host component perceived by the plant. It can be produced by non-pathogenic microbes or pathogens and participates in the ISR or SAR phenomenon, respectively (Pieterse et al., 2014). Hundreds of studies have reported, in both monocots and dicots, the ability of PGPRs, mainly *Pseudomonas, Serration* and *Bacillus*, to induce a systemic resistance toward pathogens (Pieterse et al., 2014). Elicitors plant perception usually occurs in a similar way as MAMPs or PAMPs (Newman et al., 2013; Ma et al., 2017). ISR is a promising way of pest management (Ma, Hua, et al., 2016).

Very detailed reviews of the plant immune system's molecular biology and mechanisms have been written and give a more detailed canvas of today's understanding of plant immunology (Conrath, 2011; Newman et al., 2013; Walters et al., 2013; Pieterse et al., 2014).

1.4. *Bacillus velezensis* GA1

1.4.1. *Bacillus velezensis* GA1 as model organism

This study focuses on one specific PGPR: *Bacillus velezensis* GA1. The reasons of this deliberate choice are multiple.

First, Bacilli are spore-forming, Gram-positive, rod-shaped bacteria. Bacilli are motile by peritrichous flagella and are aerobic. Some of them are capable of producing endospores that are highly resistant to stresses, making them a predilection choice for agro-industrial applications as a biocontrol agent (Sansinenea et al., 2011; Santoyo et al., 2012).

Moreover, the biocontrol market is expected to increase dramatically in the upcoming years as the worldwide compound annual growth rate (CAGR) is 8.64% (Olson, 2015). We can also notice that major companies are investing massively in biocontrol. For instance, BASF acquired Becker Underwood, while Syngenta bought Pasteuria and Devgen, and Bayer bought Agraquest (Borriss, 2015). Bacilli are key organisms of this emerging market. Indeed, nowadays, the market of PGPRs is dominated by Bacilli because, in addition to their adaptability to the agroindustrial world (i.e. endospore forming ability), some are proven to be efficient PGPRs. The operational group '*amyloliquefaciens',* along with the species *B. subtilis and B. pumilus*, gather most of the available strains (Borriss, 2015). The dominant position of *Bacillus* products underlines the importance of this genus in the PGPR sector.

Further, *Bacillus GA1* is often encountered as *B. amyloliquefaciens* GA1 (or formerly *B. subtilis* GA1 (Touré et al., 2004))[3] (Arguelles-Arias et al., 2009). However, some debates took place about the taxonomy of *B. velezensis*, *B. amyloliquefaciens subsp plantarum, B. oryzicola* and *B. methylotrophicus*. What came out is that the four species aforementioned only form one species, *Bacillus velezensis,* and that this species belongs to the 'operational' group [4] '*amyloliquefaciens*'. For instance, *Bacillus* sp. FZB42, a closely related strain belonging to the same species as GA1, is now classified as *velezensis* rather than *amyloliquefaciens subsp plantarum*. The same logic should be applied to *Bacillus* sp. GA1 and it should thus be called *B. velezensis* GA1 (Dunlap et al., 2016; Fan et al., 2017). It is worth noting that this operational group gathers most of the best PGPR Bacilli (Fan et al., 2017) and that it has undergone an evolutionary adaptation to plant associated habitat (Belbahri et al., 2017). Thus, *Bacillus* GA1 is a good representative of the *Bacillus* PGPRs.

Finally, the strain is able to produce a plethora of BSMs (Figure 1) (Arguelles-Arias et al., 2009). The BSM produced by GA1 includes non-ribosomal peptides (NRPs), polyketides (PKs), Lipopeptides (CLPs), siderophores and ribosomally synthesized peptides (Arguelles-Arias et al., 2009). Because of these numerous BSM, *B. velzensis* GA1 has an antimicrobial activity toward a large group of pathogens and thus has a great potential as a biocontrol agent (Arguelles-Arias et al., 2009).

1.4.2. *Bacillus* BSMs

1.4.2.1. NRPSs

The production of antimicrobial compounds is around 10% of *B. velezensi*s FZB42 genomes with 13 gene clusters coding for antimicrobial NRPSs indicating the crucial role of these compounds. (Fan et al., 2018).

Non-ribosomal peptide (NRPs) are metabolites synthesized by multimodular enzymes that act like an assembly line: each module is responsible for the incorporation of one amino acid, a building block into the growing polypeptide chain (Ongena et al., 2008; Argüelles Arias et al.,

[3] The GA1 strain used to be classified as '*subtilis',* however it is more related to the FZB42 strain which was classified as '*amyloliquefaciens subsp plantarum*'. GA1 was thus reclassified as '*amyloliquefaciens*' (Arguelles-Arias et al., 2009).

[4] Above species clade that includes soil-borne *B. amyloliquefaciens,* and plant associated *B. siamensis* and *B. velezensis*, the members of the clade are closely related and harbor a plant-associated life-style. This group gathers most of the best PGPR Bacilli (Fan et al., 2017).

2011). Each module can be further divided in domains, corresponding to an enzymatic unit. The modules contain at least three core domains: first, the adenylation domain selects the amino acid and transforms it into an amino acyl adenylate (by consuming an ATP). Next, the peptidyl carrier protein covalently binds the amino acyl adenylate to the synthetase. Finally, the condensation domain catalyzes the formation of a peptide bond between the amino acids and the peptide chain (Argüelles Arias et al., 2011). Interestingly, the synthesis of NRPs and PKs is allowed by the Sfp (Surfactin synthase-activating enzyme), an enzyme that transfers 4'-phosphopantheine from coenzyme A to the serine residue of the PCP domain (Fan et al., 2018). In addition to these domains, supplementary domains can be found: a thioesterase domain which releases the peptide from the synthetase, and specialized domains such as epimerization, methylation oxidation, reduction, formylation, heterocyclisation, These specialized domains provide a colossal diversity and the resultant plethora of bioactivity (Argüelles Arias et al., 2011).

Genes responsible for NRPs synthesis are usually localized in an operon (Finking et al., 2004).

- **Lipopeptides**

Lipopeptides are composed of a peptidyl backbone fused to a fatty acid. Cyclic lipopeptides (CLPs) are bacterial lipopeptides where the peptidyl backbone is cyclized. They are synthetized by non-ribosomal peptide synthetases (NRPS) organized in large clusters (Finking et al., 2004; Caulier et al., 2019). The *Bacillus* main three lipopetides classes are surfactin, fengycin, iturins (Ongena et al., 2008).

Due to their amphiphilic properties, lipopeptides usually express a high biosurfactant activity. This property induces the formation of pores and the destabilization of the membrane leading to a strong antibiosis activity (Chen et al., 2018). Moreover, LPs and CLPs are involved in motility (Raaijmakers et al., 2010; Ma, Geudens, et al., 2016), biofilm formation (Raaijmakers et al., 2006) and act as an elicitor in ISR (mainly surfactin, fengycin and iturin, (Ongena et al., 2008) and others such as Orfamide produced by *Pseudomonas* spp. (Ma et al., 2017)), and thus ISR.

- **Siderophores**

Siderophores are NRPs specialized in iron chelation. Siderophores are very diverse small molecules (500-1500 Da), water soluble and characterized by a high affinity to iron. They chelate ferric ions and deliver them into cells by active transport. The siderophores are excreted

outside the cell, where they fix the iron. The complex is then reintegrated by and into the cell. Once in the cell, the iron is either reduced or the siderophore is degraded, resulting in the solubilization of the iron (Ongena, 1996; Drehe et al., 2018).

Siderophores are known to play an important role in the competition for iron with soil-borne plant pathogens and the survival of bacteria (Ongena, 1996; Drehe et al., 2018).

1.4.2.2. PK*s*

Polyketides (PKs) are a large family of secondary metabolites that includes many bioactive compounds. The synthesis of PKs is very similar to NRPs as it is also due to the action of multimodular enzymes that performs iterative synthesis between the extender unit and the growing polyketide chain. The main difference concerns the substrate: amino acids for the NRPs and acyl-coenzyme A derivatives for the PKs. As the substrate changes, the catalytic domain changes too (Calderone et al., 2006; Borriss, 2011). As for NRPs the huge structural diversity of the PKs comes from supplementary domains of the polyketide synthetases (PKSs) such as reduction of double bounds to a saturated one or conversion of a β-keto function to a hydroxyl group (Piel, 2010).

In the interface of NRPs and PKs biosynthetic mechanisms, a series of NRPs-PKs or PKs-NRPs hybrid systems are found. The denomination these two depends on whether the backbone is composed in majority of aminoacyl or acyl-coenzymes units respectively (Argüelles Arias et al., 2011).

Interestingly, the NRPS, PKS or hybrids can often be predicted by bioinformatics tools thanks to their highly modular nature. AntiSMASH (antibiotics and secondary metabolite analysis shell) is one efficient up to date genome mining bioinformatics tool for the prediction of BSM (Blin et al., 2017). On the basis of the genome sequence, it detects BSM coding sequences and predicts the chemical structure of the BSM (Blin et al., 2017). However, when the biosynthetic product is encoded by a given gene cluster that is unknown, functional characterization of the synthase by bioinformatics methods alone is difficult or impossible (Calderone et al., 2006).

1.4.2.3. Ribosomally synthesized antimicrobials

Bacillus, among others, is also known to produce ribosomally synthetized peptides with biological activity, the so-called bacteriocins. Bacteriocins are subdivided into three classes in function of their structure and biological activity (Argüelles Arias et al., 2011). antiSMASH prediction revealed two clusters in *Bacillus velezensis* GA1 DNA that encodes for two

bacteriocins: amylocyclicin and amylolysin. Yet, they have not been detected experimentally (Figure 1).

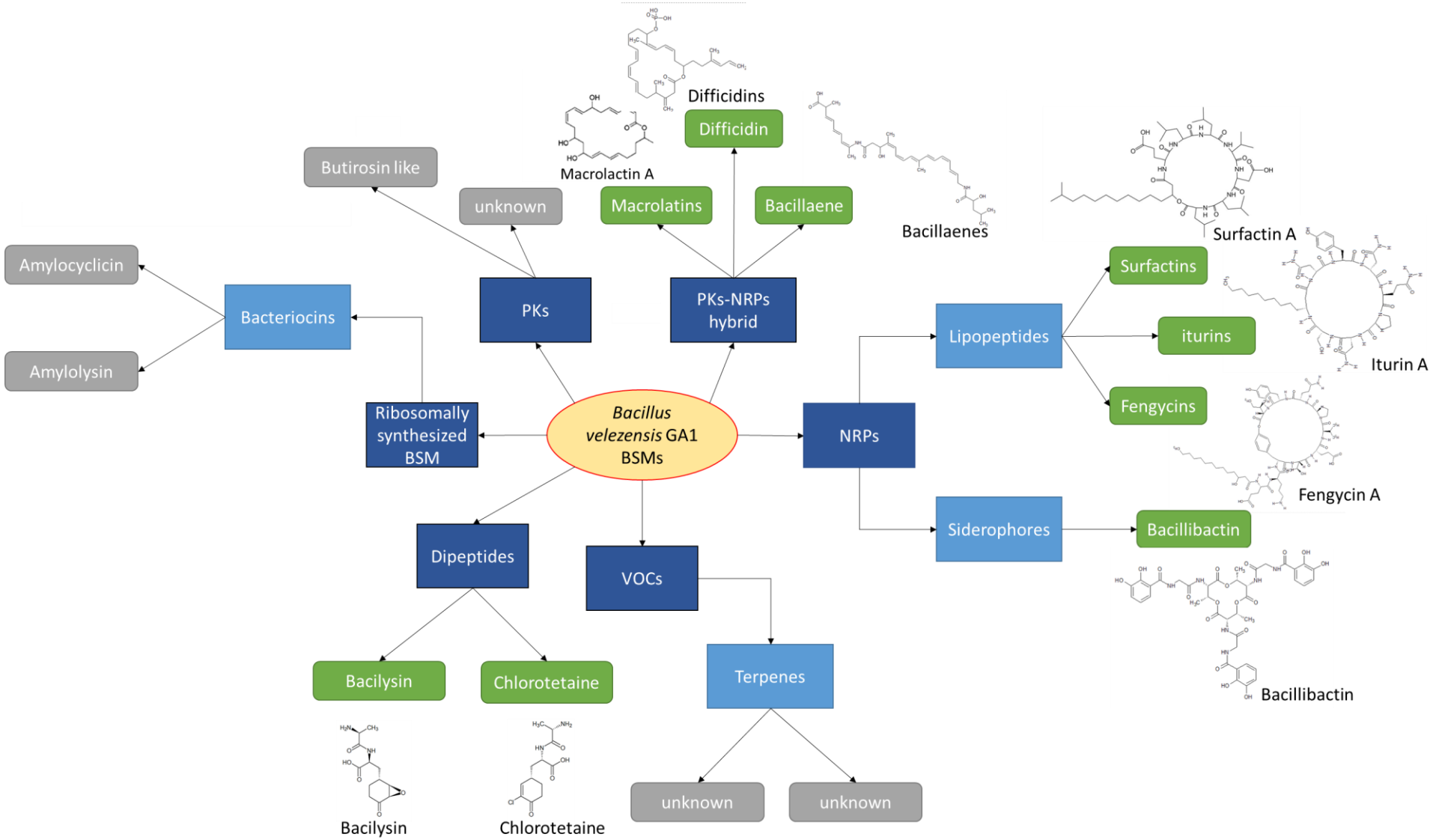


Figure 1: ***Schematic representation of the*** **Bacillus velezensis** ***GA1 BSMs****. In dark blue: BSM class; in light blue: BSM sub-classes. In Grey: BSM predicted by Antismash, In green: BSM predicted by Antismash and detected by LC-MS. The molecular structures associated with the detected metabolite is represented. In a matter of simplification, when different variants of the same molecule were encountered, only one of them is represented. The "likes" are gentic sequences that look alike the sequence of the metabolite synthesis. This representation follows the canvas proposed by (Caulier et al., 2019) but modified by scientist of the MiPI lab.*

1.5. Factors influencing *Bacillus* biocontrol agent

Nowadays, one of the challenges is to identify all the factors influencing the performances of the biocontrol agents. For instance, the host plant genotype, the interaction with biotic and abiotic factors, the previous physiologic and immune state of the plant, costs and trades-off of the use of the treatment play significant roles in the efficiency of each biocontrol agent (Walters et al., 2013). A wider and deeper understanding of these bacteria's relationship with their environment is thus needed.

1.5.1. The Rhizosphere

Lorentz Hiltner first described the rhizosphere in the early 20th century as the soil compartment influenced by the roots of growing plants. In this area, the plant plays an active role in defending itself from pathogens, orienting the microbial community and gathering nutrients. The zone is supposed to be a few millimeters thick and is 10 to 100 times richer in microorganisms (mainly composed of bacteria, about 95% of the present microorganisms) than the rest of the soil. In this area, not only is the microorganism community very diverse but small animals such as insect larvae, mites, nematodes, protozoa and amoebae can be found (Bonkowski et al., 2009; Borriss, 2015). The main rhizospheric microbiota roles are ammonifiers, nitrifiers, phytohormones producers, pathogens, antagonists (Marschner, 2012). Moreover, rhizosphere microorganisms are the main drivers of organic C, N and P turnovers and thus the recycling agents of organically bound nutrients. They have a huge impact on the availability of many nutrients like P, Fe or Mn. (through a solubilization process for phosphorus availability, the production of siderophores for iron gathering out of the environment and thanks to Red-Ox processes driven by the microorganisms for magnesium scavenging) (Marschner, 2012). Interestingly, the PGPRs' activities are modulated by the rhizosphere's biotic and abiotic factors, *in fine*, resulting in different effects on the host plant (Vacheron et al., 2013).

- **Rhizodeposits**

The rhizosphere is also characterized by a high content of carbon in comparison to bulk soil. The carbon in this area mostly comes from the plant rhizodeposits in contrast with bulk soil where most of the organic matter comes from the decay of plant remains. Rhizodeposition is the release of organic carbon by living roots into the soil. The plant is estimated to excrete 5 to 20% of its carbon by the roots; a substantial part of it reaches the rhizosphere as organic carbon, through rhizodeposition (Neumann et al., 2012).

The determination of the exact composition of the root exudates has to be considered with care due to technical reasons (the exudates are generated from plant grown in sterile conditions, far from the usual 'on the field' conditions). Nevertheless, the root exudates of sweet pepper, cucumber and tomato have been investigated and the precise data are available (Kamilova et al., 2007). In the case of the tomato, we can assume that the most important carbon sources secreted are organic acids (such as citric, malic, lactic, succinic, oxalic, and pyruvic acids), sugars (such as glucose, xylose, fructose, maltose, sucrose and ribose), amino acids, fatty acids, nucleotides, putrescine, and vitamins (Kamilova et al., 2007). Interestingly, a special group of exudate compounds are the signal molecules which are used for communication between the plant and microbes; these molecules attract specific microbial community and modulate the activities of those microbes through gene expression modulation (Kamilova et al., 2007; Vacheron et al., 2013). In this work, as the philosophy is to simulate as closely as possible the rhizosphere conditions, it was decided to grow *Bacillus* on a medium that mimics the nutritive condition the bacterium experiences while growing on a root exudate. More specifically, the medium used (Re medium) mimics the roots exudate of a tomato plant (Nihorimbere et al., 2009).

1.5.2. Abiotic parameters of the rhizosphere

The abiotic factors are crucial parameters of the efficiency of PGPR. These factors shape the soil and rhizosphere microbiome and thus determine the outcome of PGPR in the roots surrounding. Nutrient availability (C, N, P, K, Fe), soil structure and geological factors, soil water content, pH, temperature, CO_2 concentration and precipitation have been pointed out as the main factors affecting the PGPR efficiency out of the multiplicity of potent parameters (Santoyo et al., 2017). The abiotic stresses forced *Bacillus* to develop strategies to overcome these stresses. For instance, the heteromeric transporters CrcBA and CrcBB confer a resistance to the toxic fluoride ions, and McsC, McsL, McsT, McsY help it overcome osmotic stress periods (caused by drought), while SpoVAC triggers sporulation when *Bacillus* is facing severe osmotic stress (Borriss et al., 2018). Moreover, abiotic factors such as temperature, soil moisture and pH influence the production of antimicrobials by PGPRs (Raaijmakers et al., 2002). However, it is almost impossible to decipher the exact impact of each factor as they are all interconnected (Santoyo et al., 2017).

1.5.3. Biotic interactions

Plant growth promoting rhizobacteria (PGPR) and endophytic bacteria in general have developed strategies to live on and proliferate in the rhizosphere (i.e. to colonize the roots and maintain colonies in the rhizosphere). Thus, these bacteria must have co-evolved with the host plant to not only recognize a wide variety of signaling molecules, like invading pathogens related molecules, but also to respond adequately to the stimulus (like antimicrobials production) (Kusari et al., 2015).

However, most of them have not been described in detail yet as the lab conditions usually suffer from a lack of representability; most in vitro experiments use liquid monoculture, whereas in the rhizosphere and in natural environment in general, bacteria evolve in very complex communities settled in surface-associated biofilms. Each bacterium is in contact with a plethora of other microbes and larger organisms in very diverse abiotic conditions**.** This gap leads to a poor ability to accurately represent the complexity that bacteria face in their natural environments (Tashiro et al., 2013). Although in recent years the knowledge of bacterial communication has greatly improved, this field is still in its infancy (Keller et al., 2006; Kusari et al., 2015).

Nevertheless, the interactions with bacteria can be classified into 3 categories (Pierson et al., 2014):

- Interaction where none of the protagonists benefits from it (ex: bacteria living on the surface of the host (saprophytes));
- Interaction where the outcome is beneficial for both (ex: symbiotic);
- Interaction detrimental for at least one of the parties (ex: predation, parasitism,..);

Communication is one particular interaction, usually beneficial, where both interactants behave in a coordinate manner. The fact that both parties benefit from the interaction is usually seen as a key factor in the selection and of the durability of the communication. It requires two conditions to occur. First, one individual or more has to produce a signal. Second, the other organism has to perceive the signal and adapt its behavior in response (Keller et al., 2006). Communication with bacteria can be intraspecies, interspecies or even interkingdom signaling when the communicants belong to the same species, to different species of bacteria or to organisms other than bacteria respectively (Pierson et al., 2014).

Further, it is important to notice that signal perception among bacteria can happen without intended communication. Indeed, bacteria are able to detect chemicals that are produced with purposes other than communication, either when there is a chemical manipulation (the message sent gives false information) or when the communication was not intended to be understood by one species but rather by another (Keller et al., 2006).

Bacillus subtilis is able to perceive a spectrum of secondary metabolites as signals and to adapt its behavior in function (Shank et al., 2011). For instance, the perception of nystatin or amphotericin produced by *Streptomyces* enhance the matrix production while the perception of *cis*-2-decenoic acid induces a biofilm dispersal (Shank et al., 2011). This ability to finely perceive the neighboring microbial community and react correspondingly illustrates the utmost importance role of these interactions.

The understanding of cooperation behavior has greatly improved in recent years with the discovery of quorum sensing, crosstalk and electron transfer in microbial communities such as biofilm, which provides a stronger and more resilient colony-size phenotype to survive in a hostile environment (Tashiro et al., 2013).

1.5.3.1. Quorum sensing.

Quorum sensing is one of the most studied communication driven phenomena in microbiology. Quorum sensing, also referred to as cell-to-cell communication, is a phenomenon where bacteria are capable of perceiving and responding to self-generated signal molecules to coordinate their behavior in response to their population size. This action is driven by the regulation of certain gene expressions in function of the concentration of the signal molecules, usually produced by the cells themselves. The concentration of these molecules is directly linked to the cell density. The modulation of the gene expression is often characterized by a threshold in auto-inducers (signal molecules) triggering or silencing certain genes (Pierson et al., 2014; Doberva, 2016; Leach et al., 2017).

Quorum sensing is involved in many activities including colonization behavior, biofilm development, adhesion, motility, virulence, production of various enzymes, nodulation,... It can activate competition or cooperative action like antibiotic or biofilm production respectively (Pierson et al., 2014; Leach et al., 2017).

Quorum sensing can be intra or interspecies. A plethora of molecules can be involved in quorum sensing. Some of them are very specific while others can be detected by a large variety of organisms. The characteristics of the signal are the ability to be synthesized and excreted by the

bacteria, to concentrate in the environment and to be perceived by the bacteria (i.e. to interact with an intracellular receptor or on the membrane of the cell) (Pierson et al., 2014). Several QS molecular systems have been described (Pierson et al., 2014; Monnet et al., 2016). Yet, the most common Gram negative QS system is the N-acyl homoserine lactone (AHLs) also referred to as AI-1, whereas the most commonly found Gram positive communication is the oligopeptides system (Pierson et al., 2014; Monnet et al., 2015).

Interestingly, an opposite strategy, called quorum quenching, is defined as a process that inhibits quorum sensing signaling across microbial populations. This behavior works by the degradation or disruption of autoinducers. It has been shown that various organisms, from plant to bacteria, use this phenomenon. Quorum quenching is suspected to play an important role in the behavior of root-associated bacteria, both beneficial and pathogenic toward plants, potentially leading to beneficial or detrimental effects to the plant (Kusari et al., 2015; Leach et al., 2017).

Concerning the PGPRs, QS and cross-communication is often encountered among them (Pierson et al., 1998, 2014; Borriss, 2015). For instance, *Pseudomonas chlororaphis* phenazine biosynthesis is regulated by an AHL system; phenazine production is partly regulated by the PhzR/PhzI quorum sensing system. PhzI is an AHL synthase that produces the AHL C6-HSL and PhzR is the transcriptional regulator that responds to the AHL signal (Wood et al., 1996; Pierson et al., 2014). Moreover, *B. subtilis* has two well-described QS systems that enhance the production of surfactin: ComX and CSF (or PhrC). Both systems affect the phosphorylation of the ComA/ComP system. The latter modulates the surfactin production and the cell differentiation (Shank et al., 2011).

1.5.4. The importance of understanding interspecies interaction

Interest in studying rhizosphere related microbial interactions is multifaceted.

First, the co-inoculation of two or more PGPRs sometimes leads to a higher and more consistent efficiency, which can be due to a synergistic effect. For instance, a mixture of *Bacillus* SE and *Pseudomonas* KA19 showed a higher disease reduction toward *Xanthomonas campestris* than the same strains inoculated separately (Mishra et al., 2012). The authors suggest that the increase might be due to complementary modes of action. However, the mechanisms behind this type of disease control enhancement have not been investigated.

Further, research can give a better understanding of survival behavior and population dynamic on the rhizosphere. In the particular case of PGPRs, the coexistence of microorganisms, sharing

the same ecological niche as the PGPR of interest, is known to have a significant impact on its growth, morphology and even its ability to produce secondary metabolites (Bertrand et al., 2014). Moreover, chemical communication between the PGPR and the microbial community is crucial for the PGPR survival and integration (Bertrand et al., 2014). It naturally appears that understanding the crosstalk between the PGPR and the neighboring bacteria not only offers a better fundamental understanding of soil microbial ecology but also the PGPR efficiency and its outcome in the rhizosphere.

Finally, interspecies interactions can awake cryptic genes. Cryptic genes, also called orphan genes, are genes coding to enzymes and subsequently to secondary metabolites that are unknown. Many cryptic genes are found in microorganisms, in PGPR and among others. It is supposed that these genes are only expressed in specific conditions, or in the presence of a specific trigger. This pool of unknown metabolites represents a rich source of possible bioactive compounds (Gross et al., 2007). The study of interactions with co-culture systems is known to be an efficient way to awake certain cryptic genes. Yet, so far, this co-cultivation mostly involved fungi as stimulated organisms and little is known about bacteria used as such (Ochi, 2017).

1.5.5. The importance of soluble diffusible compounds on bacterial interaction

This work focuses on soluble diffusible compounds. The microbial secondary metabolites can be roughly separated into two classes: one being the volatile organic compounds, small molecules (<300 Da) from different chemical classes that have the common property to evaporate and diffuse easily, and the other being the soluble secondary metabolites, larger molecules with a higher polarity, essentially classified as NRPs, PKs, Bacteriocins, CLPs and siderophores. When the first ones act on a relatively long distance, the second ones act for a shorter distance. Interestingly, the soluble metabolites usually exhibit stronger biological activities as toxins or antibiotics (Tyc et al., 2016).

Moreover, the ‘competition sensing’ hypothesis stipulates that bacteria might be able to detect competitors because of nutrient scarcity or cellular damage (i.e. the presence of neighboring bacterial colonies) (Tyc et al., 2016). It can be easily inferred that this competition sensing is related to the presence of soluble compounds from surrounding microbes. As the aim of this work is to contribute to the general understanding of the PGPR behavior and its outcome in the

rhizosphere, it becomes natural to focus on its response to soluble metabolites as they reveal the presence of the surrounding bacterial colonies, which consitute the rhizosphere microbiome.

In addition to this consideration, antimicrobial compounds are suspected to act as signal molecules when found at subinhibitory concentrations (Tyc et al., 2016).

1.6. Choice of the interactant: *Pseudomonas* sp. CMR12a

The interactant retained is *Pseudomonas sp.* CMR12a. The choice of this particular strain is motivated by several reasons.

First of all, Pseudomonas genus was selected because it is a wide genus including aerobiquitous soil-bacteria (Doornbos et al., 2012). They are Gram-negative, aerobic, flagellated and rod-shaped bacteria (Ongena, 1996). It includes both plant pathogenic and plant-beneficial bacteria like *P. syringae* and *P. protegen*s respectively (Biessy et al., 2019). Pseudomonas bacteria are thus bacteria that are very likely to interact with *Bacillus* in the natural environment.

Moreover, the *Pseudomonas spp*. located in the rhizosphere, and among them some PGPR strains, aggressively colonize the root surface and are thus good competitors (Doornbos et al., 2012). In addition to their highly competitive behavior, they are able to produce many secondary metabolites such as cyclic lipopeptides or phenazines which are involved in the biological control of plant pathogens and plant growth promotion mechanisms in general (Meena et al., 2017; Biessy et al., 2019).

The choice of the strain *Pseudomonas* sp. CMR12a was supported by the fact that, taxonomically, *P.* sp. CMR12a is a new species that belongs to the *Pseudomonas fluorescens* complex and is positioned between the *Pseudomonas protegens* and *Pseudomonas chlororaphis* groups, two groups which contain many PGPR (Ma, Hua, et al., 2016; Biessy et al., 2019).

Next, it is a bacterium isolated from an agronomically important plant: the red cocoyam roots (*Xanthosoma sagittifolium* (L.) Schott) in Cameroon (Perneel et al., 2007). *P.* sp. CMR12a showed antagonist activity, among others, toward cocoyam and bean root associated pathogens, *Pythium myriotylum* (Perneel et al., 2007), and *Rhizoctonia solani* (De Maeyer et al., 2011; Olorunleke et al., 2015), respectively. Thankfully, *Pseudomonas* sp. CMR12a has been investigated upon its pathogenicity or phytotoxicity and showed no harmful activity toward bean and cabbage seedlings (Olorunleke et al., 2015). It is thus a good representative of the *Pseudomonas* PGPR.

In addition to this phylogenetic advantageous position, the complete genome of *P.* sp. CMR12a is available with the code 'CP027706.1'.

Finally, *P.* sp. CMR12a has the ability to produce a large variety of BSMs. Among them, the unusual ability to synthesize the sessilins and orfamides (CLPs) at the same time, two phenazines (phenazine-1-carboxylate (PCA) and phenazine-1-carboxamide (PCN), two siderophores, cyanide hydrogen,… (Perneel et al., 2007; De Maeyer et al., 2011; Biessy et al., 2019). Moreovere, numerous mutants are available (From the Laboratory of Phytophatology, Gent University)

1.7. Model plant pathogens

Plant pathogens belong to very different taxa. Indeed, they can be viruses, bacteria, fungi, nematodes, insects,… (Borriss, 2015). This work focuses on two different bacterial pathogens that are model organisms for plant pathogenic systems.

1.7.1. *Xanthomonas campestris*

Xanthomonas campestris is a Gram-negative pathogenic bacterium that belongs to the γ-subdivision of *Proteobacteria*. The taxon *X. campestris* includes more than 141 pathovars (pv.). *X. campestris pv. campestris* is a plant pathogen that causes the "black rot" disease to various crucifers (Qian et al., 2005). This pathogen is believed to be the fifth most important bacterial plant pathogen upon its scientific and economic importance (Mansfield et al., 2012). This bacterium is a model organism for the study of plant-bacteria interactions (Qian et al., 2005).

1.7.2. *Clavibacter michiganensis subsp michiganensis*

Clavibacter michiganensis is a Gram-positive bacterium among the most important bacterial phytopathogens (Mansfield et al., 2012). It is the only known representative of the genus *Clavibacter* (family *of the Microbacteriaceae). The C. michiganensis* species is subdivided in 5 subspecies in function of its host. Tomato is the characteristic host of the subspecies *C. michiganensis subsp michiganensis.* The bacterium causes significant economic losses worldwide and so far, there is no efficient treatment available beside preventive quarantine and infected plant systematic destruction (Gartemann et al., 2003).

2. Objectives

Biocontrol is often presented as an alternative to pesticides. Yet, despite the great enthusiasm of the last decades, the market of plant growth promoting rhizobacteria (PGPRs) has not boomed yet. A lack of efficiency and consistency of PGPR-based products has been pointed out as the main driver of this setback.

This poor reliability is at least partly explained by the lack of knowledge about PGPRs' behavior in their environment, the rhizosphere, making it unpredictable. In this habitat, PGPRs interact with a complex microbial community, which may determine whether the bacterium will survive, establish itself on plant roots and modulate the production of bioactive secondary metabolites (BSM).

Preliminary work performed in the MiPI lab revealed that *B. velezensis* GA1 and *Pseudomonas* sp. CMR12a, two PGPRs known for their large diversity of BSM produced, interact in a multifaceted way: on one hand, cell-free *P. sp. CMR12a* extracts appear to increase the production of *Bacillus* antimicrobial PKs (Figure 17 and 18 of the section background in annex) and, on the other hand, *P.* sp; CMR12a is able to inhibit the growth of *Bacillus* while grown on a gelified medium. Moreover, those experiments also showed a higher sensitivity of the *B. velezensis* GA1 mutant hampered in surfactin production toward *Pseudomonas* inhibitory effect.

This work aims to further understand this complex interaction by identifying the BSM of *P.* sp. CMR12a that triggers the production of antibacterial PKs by *B. velezensis GA1* and to identify the compound secreted by *Pseudomonas* responsible for growth inhibition of *B. velezensis* GA1.

In order to achieve these objectives, *Bacillus* cells were cultivated on a medium that mimics the root exudates (i.e. the nutritive conditions experienced by in its natural niche) to be as close as possible to the natural conditions encountered on the plant root surface. Moreover, both *Pseudomonas* and *Bacillus* bacteria were grown in liquid medium as this allows more accurate measurements of biomass and production rate, easier sampling of culture supernatant (and subsequently the BSMs produced) and as it lowers matrix interferences.

Chapter 2: Materials and methods

1. Strains and growth conditions

The strains used in this study are presented in table 1. *Pseudomonas* strains were routinely grown at 30°C, 160rpm on CAA medium (10g of casamino acids, 0.3g of K_2HPO_4, 0.5g of $MgSO_4$ per litter of water, pH 7) supplemented when appropriate with 20µg/l of $FeCl_3.6H_2O$. For specific experiments *Pseudomonas* strains were grown in LB medium (10g of tryptone casein, 5g of yeast extract, 10g of NaCl (and 14g of agar for solid medium) on 1l of water, pH 7), KB medium **(** 20g of bacteriological peptone, 10g of glycerol and 1.5g of K_2HPO_4, 1.5g of $MgSO_4.7H_2O$ per litter of water**,** pH 7) or RE medium composed of 1/2 l of all medium (0.685g of KH_3PO_4, 21g of MOPS, 0.5g of $MgSO_4.7H_20$, 0.5g of KCl, 1.0g of yeast extract and 100µl of the trace solution (120mg of $Fe_2(SO_4)_3$, 40mg of $MnSO_4$, 160mg of $CuSO_4$ and 400mg Na_2MoO_4 per 10ml) and 1/2l of tobacco medium **(**2.0g of glucose, 3.4g of fructose, 0.4g of maltose, 0.6g of ribose, 4.0g of citrate, 4.0g of oxalate, 3.0g of succinate, 1.0g of malate, 10g of fumarate, 1.0g of casamino acids, 2g of $(NH_4)_2SO_4$ per liter**,** pH 7). The *Bacillus velezensis* strains were grown on RE medium, 30°C 300rpm.*Xanthomonas campestris* and *Clavibacter michiganensis* were cultivated on LB medium.

2. Supernatants preparation

2.1. *Pseudomonas* supernatant

Fresh colonies of *Pseudomonas* sp. strains cultured on LBA medium at 30°C during 24h were collected, washed three times with CAA medium and centrifuged at 5000rpm for 5min. The bacteria were then placed in the 250ml flasks with 100ml of liquid medium with a final OD of 0.05. The bacteria were then incubated at 30°C for 48h with 160rpm orbital shaking. After incubation, *Pseudomonas* sp. cells were centrifuged for 15min at 8000rpm. Finally, the supernatants were sterilized with a 0.22µm filtered and were then stored at -20°C

2.2. Coculture supernatant

In all the coculture experiments, the *Bacillus velezensis* bacteria were collected from fresh LBA plates and washed with the same protocol as *Pseudomonas* sp. *Bacillus velezensis* GA1 cells were grown on RE medium with an initial OD of 0.1 with 2% (v/v) of *Pseudomonas supernatant.* The experiments were run on microplates (24 wells) (V_{wells}=2ml) (VWR

international sprl, Leuven, Belgium).The strains used are summarized in Table 1. Each coculture set up was performed in triplicate.

3. Supernatants fractioning/concentration

The secondary metabolites of the CMR12a supernatants are concentrated with a C18 cartridge 'Chromafix, small'(Macherey-Nagel, Düren, Germany). The column is conditioned with 10ml of MetOH followed by 10ml of milliQ water. Then, 20ml of supernatant is flowed through the column. The metabolites are eluted with 0.5ml of solution of growing acetonitrile (ACN)/water ratio.

4. Iron Dosage

The Iron dosage was performed with VISTA-MPX ICP-OES (Varian, Palo Alto, CA, USA) (Induced coupled plasma-Optical emission spectrometry)with a CCD detector. The signal was measured at two different wavelengths: 234.350nm and 261.187nm.

Table 1: ***Strains used in this work***

Strains	Description	Reference	Collection of origin
Bacillus velezensis			
GA1	wild type	Touré et al. 2004	Laboratory of MiPI, Uliege
ΔSrf	GA1 disrupted of *srfaA* gene; cat^R; unable to produce surfactins	Unpublished strain (MiPI)	Laboratory of MiPI, Uliege
Pseudomonas sp.			
CMR12a	wild type	Perneel et al., 2007	Laboratory of Phytopathology, Ghent University
Δsess	CMR12a disrupted of *sesA* gene; Gm^R; unable to produce sessilins	D'aes et al., (2011)	Laboratory of Phytopathology, Ghent University
Δorf	CMR12a deleted of *ofaB* and *ofaC* genes; Gm^R; unable to produce orfamide	D'aes et al., (2014)	Laboratory of Phytopathology, Ghent University
Δphz	CMR12a deleted of phenazine biosynthesis operon; unable to produce phenazzine	D'aes et al., (2011)	Laboratory of Phytopathology, Ghent University
Δsess-orf	CMR12a disrupted of *sesA* gene and deleted of *ofaB* and *ofaC* genes; Gm^R; unable to produce sessilin and orfamide	D'aes et al., (2014)	Laboratory of Phytopathology, Ghent University
Δsess-phz	CMR12a disrupted of *sesA* gene and deleted phenazzine biosynthesis operons; Gm^R; unable to produce sessilin and phenazzine	D'aes et al., (2011)	Laboratory of Phytopathology, Ghent University
Δorf-phz	CMR12a deleted of *ofaB* and *ofaC* genes and phenazzine biosynthesis operons; unable to produce orfamide and phenazzine	D'aes et al., (2014)	Laboratory of Phytopathology, Ghent University
Δsess-orf-phz	CMR12a disrupted of *sesA* gene and deleted of *ofaB* and *ofaC* genes and phenazzine biosynthesis operons; Gm^R; unable to produce sessilin, orfamide and phenazzine	D'aes et al., (2014)	Laboratory of Phytopathology, Ghent University
2.95	CMR12a deleted of *hemN* gene; unable to produce pyoverdine	Unpublished strain	Laboratory of Phytopathology, Ghent University
COW 8	wild type	Oni et al., 2019	Laboratory of Phytopathology, Ghent University
COR 58	wild type	Oni et al., 2019	Laboratory of Phytopathology, Ghent University
COR 33	wild type	Oni et al., 2019	Laboratory of Phytopathology, Ghent University
Pseudomonas tolaassii			
CH36	wild type	Rokni-Zadeh et al., 2012	Laboratory of Phytopathology, Ghent University
Xanthomonas campestris pv. campestris	wild type	DSMZ[1] N°3586	Laboratory of MiPI, Uliege
Clavibacter michiganensis subsp. michiganensis	wild type	DSMZ[1] N°20741	Laboratory of MiPI, Uliege

5. Chromatography analysis and HPLC purifications

5.1. *(*dihydro)bacillaene and (oxy)difficidine analysis.

The analysis of the (dihydro)bacillaene and of the (oxy)difficidin content was performed by UPLC/MS using an Acquity UPLC® BEH C 18 column (L=50mm, D= 2.1mm, Particles diameter= 1.7µm) (Waters, milford, MA, USA). The volume injected was 10µl. The elution program is described in table 2. Mass spectrometer (Acquity UPLC ® Class H SQD (Water, milford, MA, USA)) was set in negative mode ESI- (cone voltage: 60V). A single ion recording (SIR) method was used for the four characteristic molecular ions of these compounds: 543.5 ($[m-H]^-$ of difficidin), 559.5 ($[m-H]^-$ of oxydifficidin), 579.5 ($[m-H]^-$ of bacillaene) and 581.5 ($[m-H]^-$ of dihydroxybacillaene).

*Table 2:**Elution program of the analysis of (dihydro)bacillaene and (oxy)difficidin.***

Time (min)	Flow (ml/min)	% H2O	%ACN
0	0.600	70	30
5	0.600	50	50
5.5	0.600	5	95
7.5	0.600	5	95

5.2. Pyoverdines analysis

The same column and detector were used for this method as in the method for the analysis of (dihydro)bacillaene and (oxy)difficidin. The elution program is given in table 3. The volume injected was 10µl. The ionization mode was both ES- and ES+ with and mass range from m/z 250.00 to 1400.00. The cone voltage was 120V.

*Table 3: **Elution program of the analysis of Pyoverdines***

Time (min)	Flow (ml/min)	%H_2O	%ACN
0	0.350	100	0
5	0.350	55	45
5.5	0.6	5	95
6.7	0.6	5	95

5.3. *Bacillus* PKs-NRPs broad analysis

The same column and detector were used for this method as in the method for the analysis of (dihydro)bacillaene and (oxy)difficidin. The elution program is given in table x. The volume injected was 10µl. The ionization mode was both ES- and ES+ with mass range of from m/z 300.00 to 2048.00. The cone voltage was 60V.

Table 4: ***Elution program of the analysis of the PKs-NRPs broad analysis***

Time (min)	Flow (ml/min)	%H_2O	%ACN
0	0.6	85	15
2	0.6	85	15
7	0.6	5	95
9.5	0.6	5	95

5.4. Siderophore purification

Pyoverdines and pyochelin were purified in two steps. First, 90ml of supernatant was loaded on a C18 cartridge 'Chromafix, large' (Macherey-Nagel, Düren, Germany) (previously conditioned with 20ml MetOH followed with 10ml mQ water) and was eluted with 3 times 2ml of a solution of water and ACN (15, 30, 50% of ACN (v/v)). Secondly, the fractions were injected on HPLC for purification with performed on an eclipse+ C18 column (L=150mm, D= 3.0mm, Particles diameter 5µm) (Agilent, Waldbronn, Germany). The elution program is summarized in table 5. The volume injected was 100µl. The UV-Vis absorbance was measured with a VWD Agilent technologies 1100 series (G1314A) detector (Agilent, Waldbronn, Germany). The lamp used was a Deuterium lamp G1314 Var Wavelength Det. (Agilent, Waldbronn, Germany). Two wavelengths were selected: 320 and 380nm. 320nm was used for the detection of pyochelin and 380nm was used for the detection of pyoverdines. The fractions containing the pyoverdines and pyochelin were collected directly at the detector output.

Table 5: ***Elution program of the Siderophore purification method***

Time (min)	Flow (ml/min)	%H_20	%ACN
0	1.0	100	0
2	1.0	100	0
10	1.0	85	15
12	1.0	45	55
17	1.0	5	95
20	1.0	5	95

5.5. Pyoverdine and pyochelin identification, quantification and purity estimation

The same column and this method as in the method for the analysis of (dihydro)bacillaene and (oxy)difficidin. The elution program is given in table 6. The volume injected was 20µl. Two detectors were used: a diode array detector (DAD) 190 to 601 nm (steps: 1nm) and a Q-TOF (tandem mass spectrometry, quadrupole and Time of flight detector combined) (Agilent, Waldbronn, Germany). Electrospray ionization was performed in positive mode (ESI+) (Dual AJS ESI) (Vcap= 3500V, Nozzle Voltage= 1000V), with mass range from m/z 200 to 1500.

Table 6: ***Elution program of pyoverdines and pyochelin identification, quantification and purity estimation.***

Time (min)	Flow (ml/min)	% H2O	%ACN
0	0.300	100	0
6	0.300	100	0
11	0.300	0	100
13	0.300	0	100

- **Pyoverdine Collision**

The pyoverdine collision used the same chromatographic method and instrument and the same analytical method and instrument except for the collision chamber which is an hexapole positioned between the quadrupole and the time of flight detector (Agilent, Waldbronn, Germany). The ions 1288.58 $[m+H]^+$ is selected. The collision energy is 75V.

5.6. *Pseudomonas* BSM broad analysis

The same column and UV-Vis and mass spectrometry detectors were used as in the method 5.5.'pyoverdine and pyochelin identification, quantification and purity estimation'. The volume injected was 20µl. The elution program is given in Table 7. The mass analysis was performed in positive mode ES+ (Dual AJS ESI) (Vcap= 3500V, Nozzle Voltage= 1000V), with a mass range from m/z 180 to 1700.

Table 7: ***Elution program*** **Pseudomonas** ***BSM broad analysis***

Time (min)	Flow (ml/min)	%H_2O	%ACN
0	0.450	90	10
20	0.450	0	100
22	0.450	0	100

6. Inhibition tests in liquid

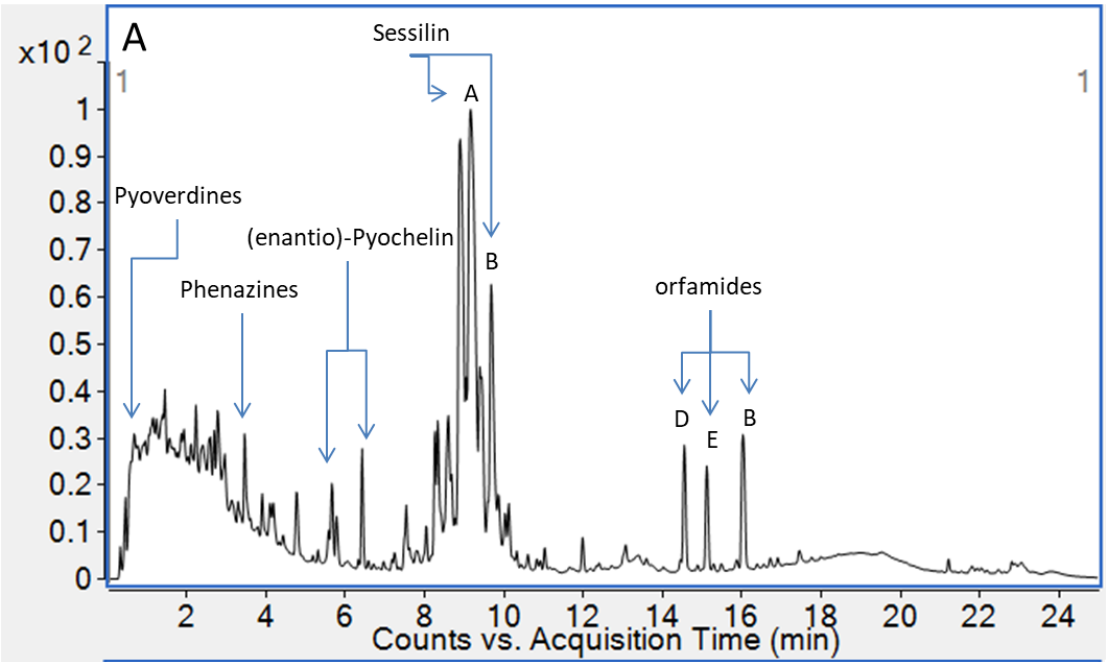
The antimicrobial activity of *B. velezensis* GA1 on the following phytopathogenic strains *X. campestris pv. campestris* or *C. michiganensis subps. Michiganensis* and the antimicrobial activity of *Pseudomonas* sp. CMR12a supernatant on *B. velezensis* GA1 were performed in liquid medium. The cells were collected from fresh culture, were washed 3 times and they were suspended in liquid medium (LB and RE for *X. campestris pv. campestris s* and *B. velezensis* GA1 respectively), centrifuged 10min at 8000rpm and adjusted to an OD of 0.125 in appropriated medium (LB and RE for *Xanthomonas* and *Bacillus* respectively). For *Xanthomonas,* the culture was performed a 96 wells microplate. The initial OD was 0.1 in LB medium and 1, 2 or 5% (v/v) of sterile coculture supernatants were added on the wells. For *Bacillus*, on a 24 wells plate, 2ml of *Bacillus* suspension at an OD of 0.125 were added with 6% (v/v) of supernatant of the mutant of *Pseudomonas* sp. CMR12a.

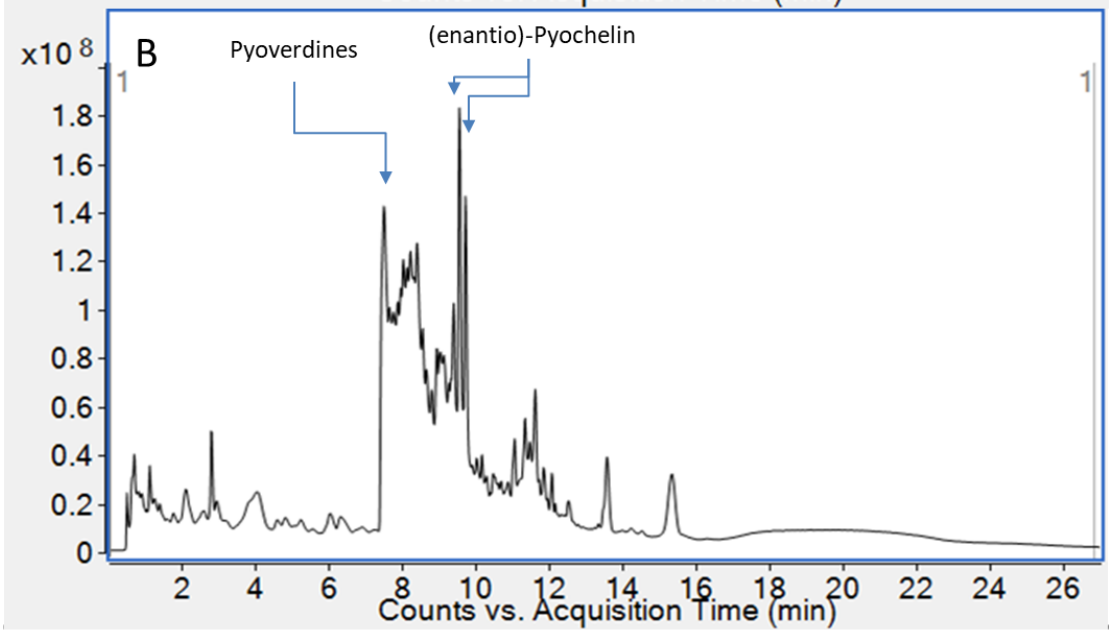
The OD was measured every 30min during 24h with a Spectramax® (Molecular Devices, Wokingham, UK), continuously shaken, at 30°C.

Chapter 3: Results

1. *Pseudomonas* sp. CMR12a BSMs

As stated earlier, the *Pseudomonas* sp. CMR12a has been selected for interspecies interactions with *Bacillus* notably because of its potential to produce a wide range of bioactive secondary metabolites, the availability of genome information and the adequate mutants of the strain. It is essential to have a profound knowledge of the *P.* sp. CMR12a BSMs to apprehend the *Bacillus*-*Pseudomonas* interaction at a molecular level (Bertrand et al., 2014). Based on the annotated genome, we performed the antiSMASH analysis for BSM prediction. We have also developed UPLC-MS(MS) methods to detect the metabolites produced. The BSMs produced in CAA medium were analyzed as illustrated in Figure 2. The identification of metabolites was based on the analysis by UPLC-qTOF MS/MS based on the exact mass (Figure 2) and confirmation with mutants when available (Table 1). Five main BSMs and several variants were identified based on previous studies as orfamides (D'aes et al., 2014), sessilines (D'aes et al., 2014), phenazines (Perneel et al., 2007), pyochelins (Drehe et al., 2018) and pyoverdines (Hartney et al., 2013). The pyoverdines and pyochelin structure have not been described in detail for *P.* sp. CMR12a. So, we based our research on the siderophores of the closely related strain *P.protegens* Pf-5. Two methods were used to accurately detect and characterize all the metabolites described in Figure 2: a general one and a specific one for the detection of the siderophores (pyoverdine and pyochelin). Figure 5 maps the different BSMs of *P.* sp. CMR12a. As one can notice, only a few of all the potential metabolites were detected and the production of many more has to be awaken. Indeed, *P.* sp. CMR12a has the genetic potential to produce a large variety of BSM (8 different NRPs, 2 bacteriocins, 1 AHL, 1 β-lactam, …).

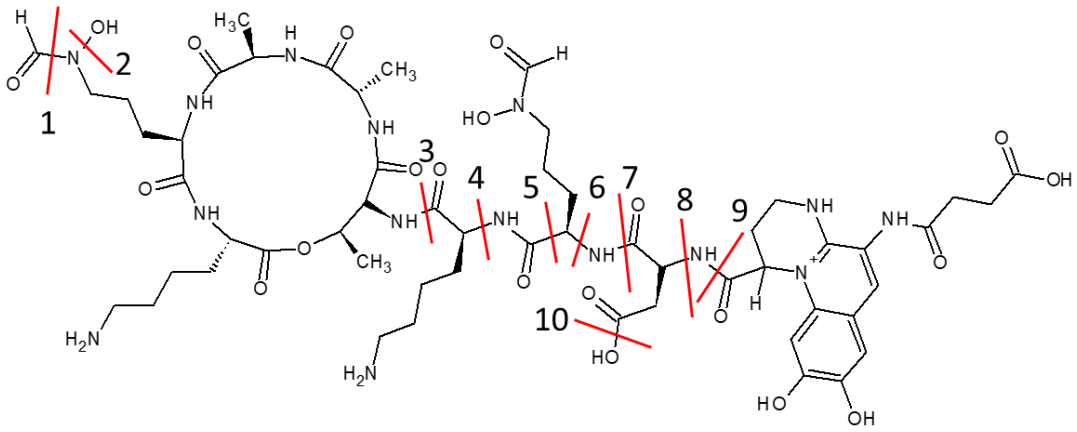




Metabolite	Variant	m/z
Sessilin	A	1014.6270
	B	992.6139
Orfamide	B	1281.8280
	D	1253.7967
	E	1279.8123
Phenazine	Phenazine-1-carboxylate	225.0818
	Phenazine-1-carboxamide	224.0818
Pyoverdine	-	645.2973
Pyochelin	Pyochelin	325.0675
	Enantio-pyochelin	325.0675

Figure 2: ***Pseudomonas sp. CMR12a mass chromatograms analyzed with UPLC-QTOF and annotated with the identified metabolites****. A:chromatogram of the general method. B: chromatogram of the siderophore specific method.*

Among the different metabolites detected, pyoverdine was the only one whose structure has not yet been described for *P* sp. CMR12a**.** Pyoverdines can harbor many different structures; pyoverdine structure is very variable among species and even within a species, among strains. It is not uncommon to find strains with their own specific pyoverdine (Visca et al., 2006). The precise identification of the *P*. sp. CMR12a pyoverdine revealed that it had two different forms (m/z= 1288.5813; m/z 1335.6159) (section 2. of the annex). We elucidated the structure of the 1288.5813 variant based on the exact mass and its fragmentation. The structure of the molecule with the fragments and their associated m/z ratio is represented in figure 3.



FRAGMENTS	m/z	FRAGMENT INTENSITY
1	1260.5857	2980.48
2/10	1270.5686	2025.38
3	759.2935	1620.38
4	648.2229	944.03
5	603.2024	829.93
6	490.1537	716.68
7	473.1280	21347.25
8	375.1266	2453.58
9	358.1015	1312.32
10	1270.5686	2025.38

Figure 3: ***Fragmentation pattern of pyoverdine corresponding to the molecular ion m/z ion 1288.5913 by MS-MS.****.* *Fragmentation performed at 75V.*

The two variants of the *Pseudomonas* sp. CMR12a pyoverdine had the same exact mass and retention time as the ones produced by *P. protegens* Pf-5. Moreover, the structure corresponding to the variant m/z 1288.5913 was exactly the same as the one described for *P. protegens* PF-5 (Hartney et al., 2013). We then compared the pyoverdine biosynthesis cluster of both strains (Figure 4). The comparison revealed a high level of identity among the two clusters.

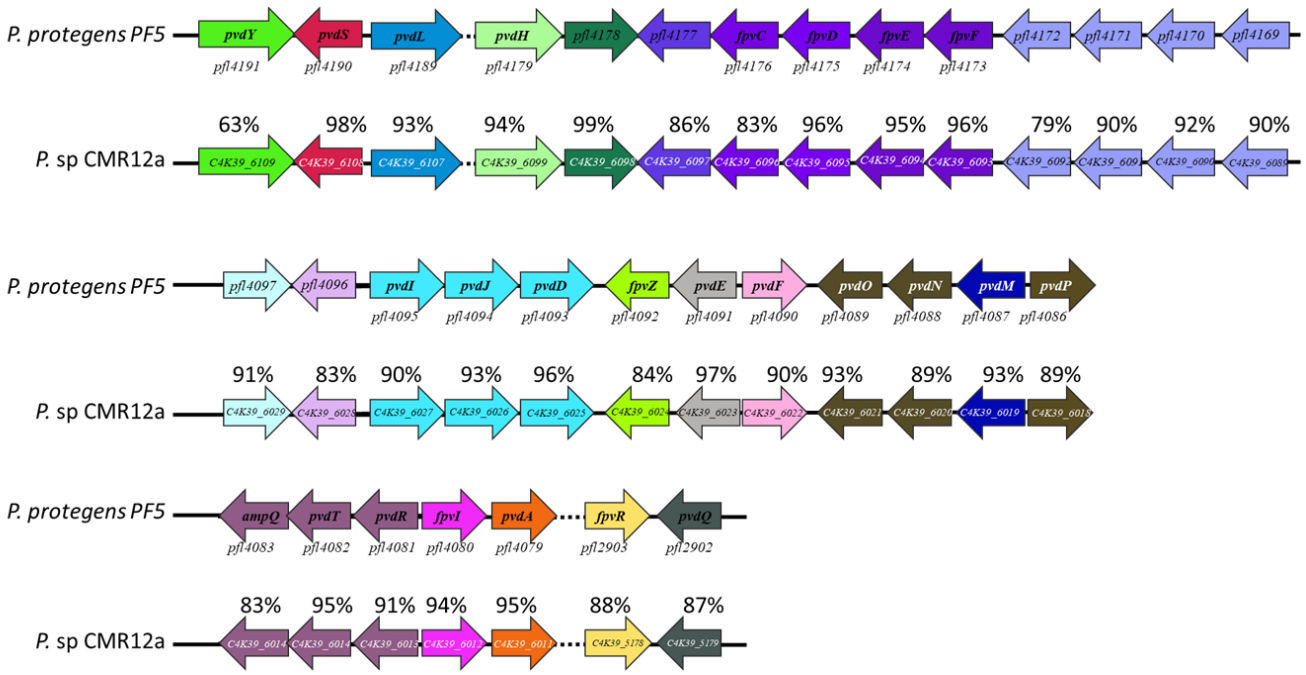


*Figure 4:**Genes identity of* **Pseudomonas** *sp. CMR12a and* **Pseudomonas protegens** *Pf-5pyoverdine clusters*
*(*Pseudomonas protegens *Pf-5 clustered was described by (Hartney et al., 2013))*

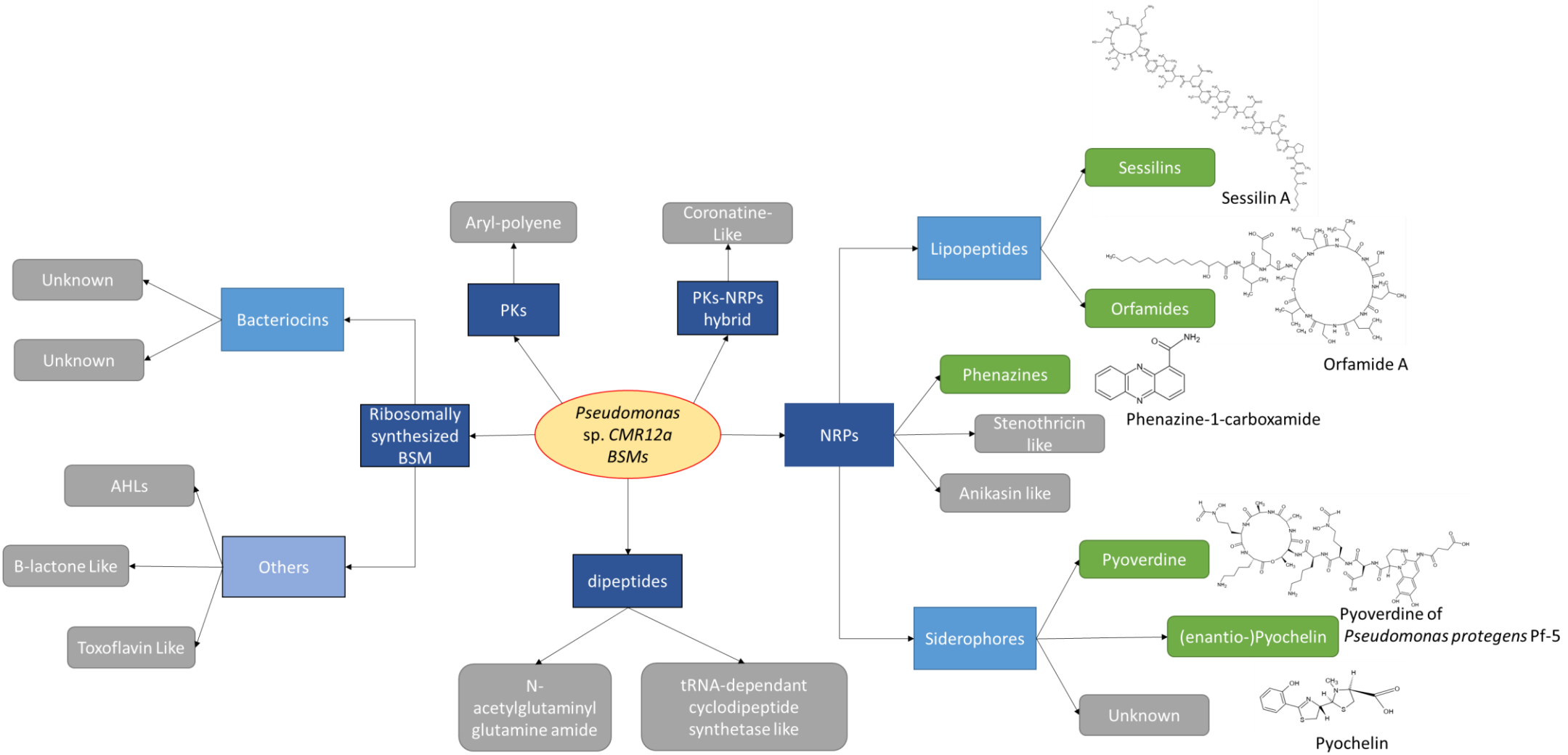


Figure 5: **Pseudomonas *sp. CMR12a BSMs***. *In dark blue: BSM Class. In light blue: BSM sub-classes. In grey: BSM predicted by antiSMASH but not detected by UPLC-MS. In green, BSM detected by UPLC-MS. The detected BSMs are illustrated by their molecular structure. In a matter of clarity, only one of the variants of a same molecule is represent.*

2. The interplay between CLPs drives the antagonistic interaction between the two bacteria

2.1. Sessilin is the main *Pseudomonas sp.* CMR12a metabolite retaining antibacterial activity against *Bacillus velezensis* GA1

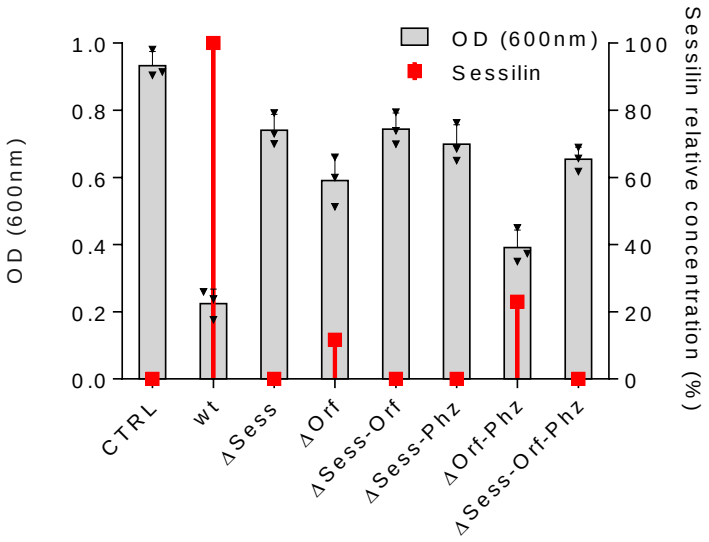


Figure 6: ***Involvement of sessilin in growth inhibition of*** **Bacillus velezensis** ***GA1****. The optical density* of B. velezensis *GA1 is measured after 7h culture while 6%(v/v) of the supernatant of* Pseudomonas *sp. CMR12a mutants is added to the* Bacillus velezensis *GA1 culture. CTRL: control group (i.e.* Bacillus velezensis *GA1 grown without* Pseudomonas *supernatant). Wt: wild type. ΔSess: mutant impaired in sessilins synthesis Δorf: mutant impaired in orfamides synthesis,. ΔPhz: mutant impaired in phenazine synthesis. The sessilin relative concentration is express as % of the wild type.*

As developed in section 1., the *P.* sp. CMR12a BSM screening revealed multiple metabolites with antibacterial activity and thus potentially inhibitory for *Bacillus*. However, according to preliminary tests to this work (Figure 19, section 1. of the annex), sessilin, one of the two CLPs actively produced by the strain, seems to be the major active compound responsible for toxicity against *Bacillus*. Indeed, liquid culture experiments allowed us to have a more detailed study of the effect and revealed a clear correlation between the sessilin content of the supernatant and the growth inhibition (Figure 6). This observation suggests that other metabolites may be involved in *Bacillus* inhibition. The deletion of orfamide and phenazine, two metabolites known for their antimicrobial activity is not linked to a loss of inhibitory activity.

2.2. Surfactin counteracts the toxic effect of sessilin and renders *B.velezensis* GA1 less sensitive to *Pseudomonas*

We observed the formation of a white line between *Pseudomonas* and *Bacillus* colonies when grown close together in gelified medium (Figure 7.A). The WLIP (white line inducing principle) phenomenon occurs between two lipopeptides that interact together and form a white line when grown in agar plate. It was first described between the white line inducing principle, a lipodepsipeptide produced by *Pseudomonas* reactants and the tolaasins of *P. tolaasii* isolates. (Mortishire-Smith et al., 1991). Moreover we found that both surfactin and sessilin were involved in the phenomenon as no white line was observed when the interactants were unable to produce at least one of these metabolites (Figure 7.B,C,D). Our hypothesis was that surfactin interacts with sessilin, lowering the sessilin toxicity.

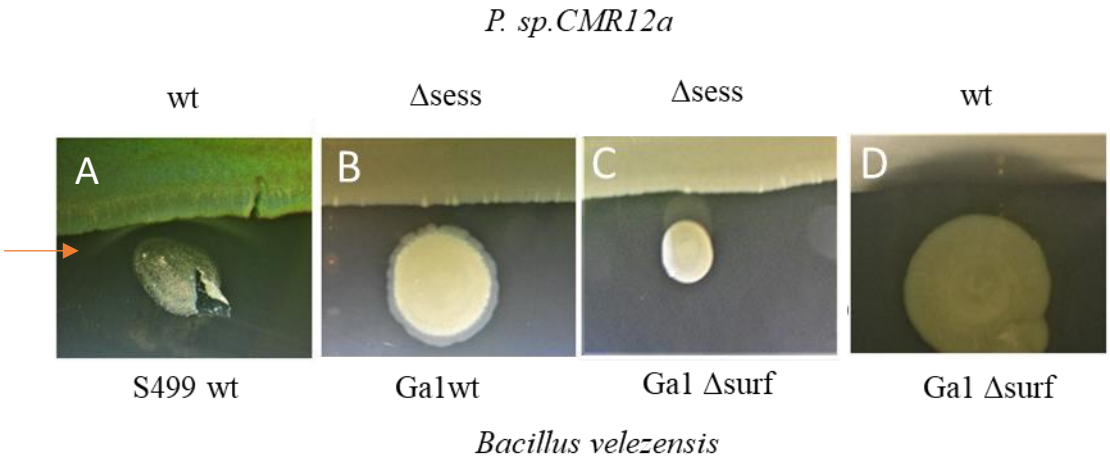


Figure 7: ***White line formation between*** **Bacillus velezensis spp.** ***and*** **Pseudomonas** ***sp. CMR12a depends on the ability of*** **Bacillus** ***to produce surfactin and of*** **Pseudomonas** ***to produce sessilin.*** *The experiment was performed on gelified RE medium. S499:* Bacillus veezensis *S499 wt: wild type. Δsess:* Pseudomonas sp. CMR12a *mutnt unable to produce sessilin. GA1 Δsurf:* Bacillus velezensis GA1 *mutants unable to produce surfactin MiPI lab, unpublished. The red arrows indicates the position of the white line*

To test this hypothesis, we compared the sensitivity of the surfactin mutant to the wild type in in terms of growth in liquid culture (figure 8). Moreover, a chemical complementation was performed by adding 10µM of pure surfactin (amount in the range of surfactin production by the wild type after 6h of growth).

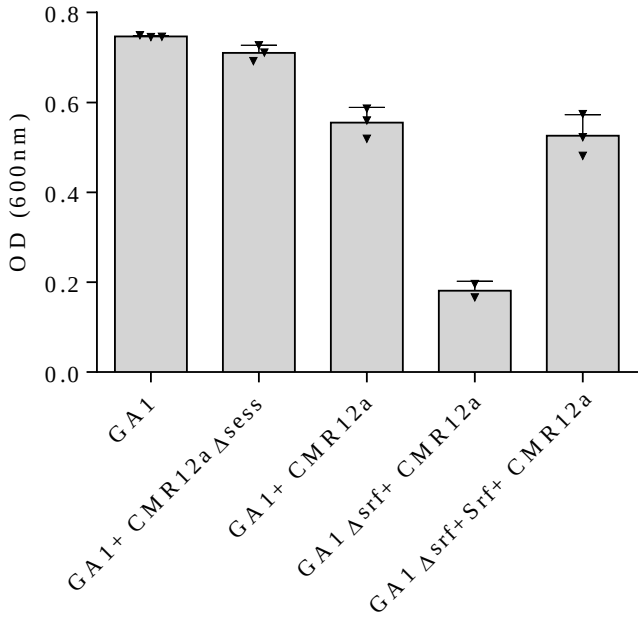


Figure 8: ***Impact of the surfactin on the sesnsitivity of*** **Bacillus velezensis** ***GA1 toward*** **Pseudomonas** ***sp. CMR12a antimicrobial activity****. The optical density of* Bacillus velezensis *GA1 is measured after 10h culture (GA1: wild type strain; GA1ΔSrf :*B. velezenesis *GA1 deleted with surfactin production gene; +CMR12a: sample with addition of 4% (v/v) of* Pseudomonas *sp. CMR12a supernatant after 6h of culture of* Bacillus*; +Srf: addition of 10µM of surfactine to the* Bacillus *culture after 6h).*

Figure 8 shows that sessilin is the main metabolite in *Pseudomonas* sp. CMR12a supernatants retaining activity toward *B. velezensis GA1* as the supernatant of the *P.* sp. CMR12a mutant (repressed in sessilin production) shows no differences with the controls. This graph also shows that the surfactin mutants are more sensitive toward sessilin; wild type *B. velezensis* GA1 shows a stronger resistance toward sessilin (i.e. a higher OD). Interestingly, this resistance can be restored by supplementing the medium with surfactin. We thus conclude that surfactin protects the *Bacillus* cells from the sessilin toxicity by reacting together to form an insoluble precipitate (white line).

3. Identification of the *Pseudomonas* compound(s) stimulating the synthesis of antibacterial polyketides in *Bacillus velezensis* GA1

Preliminary results revealed that *Bacillus velezensis* GA1 antimicrobial activity was enhanced by the presence of *Pseudomonas* and in presence of soluble metabolites of *Pseudomonas* sp. CMR12a (s) to the growth medium (Figure 16 and 17 and, Section 1. on the Annex). This largest part of our work was devoted to the identification of the triggering molecules secreted by *Pseudomonas* sp. CMR12a.

3.1. Stimulation of dihydrobacillaene as best proxi of the *Bacillus* response

We first measured the increase in production of the four PKs involved in the antibacterial activity of *B. velezensis* GA1 at three different timepoints following supplementation of the culture medium with *Pseudomonas* sp. CMR12a supernatant collected after cultivation in CAA medium (figure 9). The increase of dihydrobacillaene production at 24h appeared to be both the highest (about 500%) and the most consistent in comparison with the other PKs and timepoints. We therefore selected dihydrobacillaene production after 24h as a marker of *Bacillus* response in the following experiments.

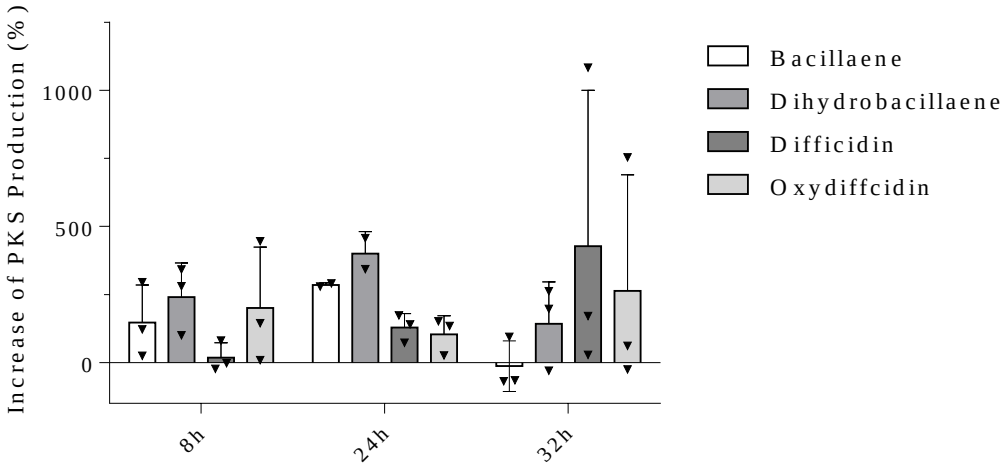


Figure 9: ***Effect of* Pseudomonas *sp. CMR2a supernatant on the production of PKs (bacillaene, dihydrobacillaene, difficidin and oxydifficidin) by* Bacillus velezensis *GA1 at three different timepoints*** *(8h,24h,32h).*

3.2. Siderophores are the main *P. sp.* CMR12a compounds stimulating PKs synthesis by *B. velezensis* GA1

3.2.1. The most active *B. velezensis* CMR12a culture supernatants are enriched in siderophores

Pseudomonas sp. CMR12a was grown on five different media following the OSMAC method (One strain, multiple compounds) to induce or boost the production of certain specific metabolites or wake some cryptic genes (Ochi, 2017). We evaluated the impact of the culture medium on the BSM production by the strain and on the triggering activity of the corresponding supernatants (Figure 10).

Each medium was chosen for a specific reason. First, the root exduate medium (RE medium) is supposed to mimic the nutritional sources found on the rhizosphere as it is formulated to resemble the root exudates of tomato plants as described by Kamilova et al., (2007). The carbon comes from sugars, organic acids and a few amino acids whereas nitrogen is mostly present as ammonium (NH_4^+) on this medium. Next, the casamino acid medium (CAA medium) was chosen. It is composed mainly of amino acids and small peptides. These compounds are at the same time the sources of carbon and nitrogen. This medium is known to enhance the production of pyoverdine due to its low iron content (Popat et al., 2017).The third medium chosen was the Luria-Bertani medium (LB medium) as it is a classically used laboratory medium and it contains peptone as main nutrients. The fourth one was the King B medium (KB medium) as it is optimized for *Pseudomonas* growth and secondary metabolite production. The main carbon source is glycerol and the nitrogen comes from peptone on this medium. KB medium is known to favor the production of phenazines and siderophores (Haggag et al., 2012; Ntyam et al., 2018). Finally, iron supplemented CAA medium (CAA+Fe) was tested. It is essentially the same as the CAA medium except that it is supplemented with iron ($FeCl_3$) to repress the production of siderophores (Ongena, 1996).

As expected, the production of siderophores (pyoverdine and pyochelin) is inhibited when the iron concentration in the medium is high enough (Ongena, 1996) (Figure 10). Indeed, the fresh CAA medium, which induced the best siderophore production, has an iron concentration below LOQ (Table 8., section 8., on annex) while the RE medium, which does not induce much siderophore production has a high iron concentration of 0.093 mg/l. Moreover, the iron supplemented CAA medium does not induce the production of siderophores. The inhibition of

pyoverdine and pyochelin production is illustrated in figure 24 in section 3 of the annex with the UPLC-MS chromatograms of the supernatants generated in both CAA and CAA+Fe media.

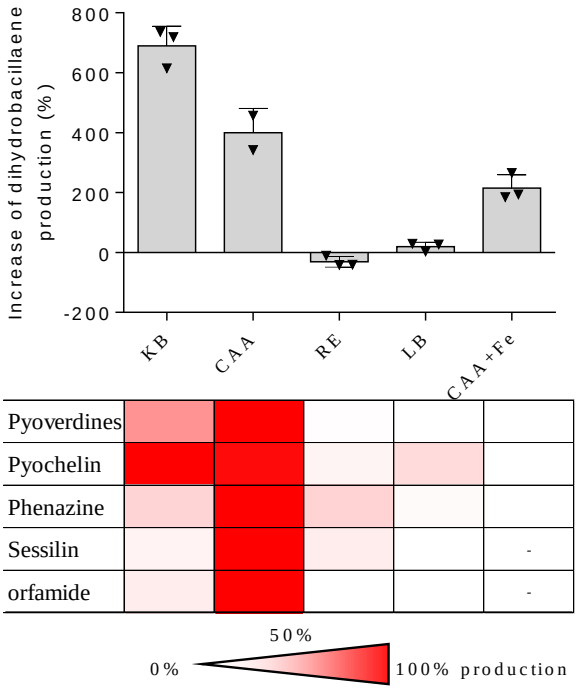


Figure 10: ***Differential triggering activity and BSM production of*** **Pseudomonas** ***sp. CMR12a in function of the culture medium****. Five different media were tested: KB, CAA, RE, LB and iron supplemented CAA medium (CAA+Fe). In the coculture, 2% (v/v) of CMR12a supernatant was added to the* Bacillus velezensis *GA1 culture. The iron supplemented CAA medium (CAA+Fe) does not contain sessilins and orfamides as the double mutant* Pseudomonas *sp. CMR12a Δsess-orf was used for that assay. The relative concentrations of pyoverdines and pyochelin were estimated by pic areas ratio of the DAD at 380 and 320nm respectively and the relative concentration of sessilins, orfamides and phenazines were estimated by pic areas ratio of the specific m/z of the metabolite. The value '100% production' corresponds to the maximum production observed among the 5 culture conditions*

In Figure 10, the relative quantification of BSMs is linked to the ability of the supernatant generated in each medium to induce a boost of dihydrobacillaene production. Interestingly, KB and CAA media, which happened to be the best BSM producers, are more efficient than RE, LB and iron supplemented CAA media.

A more detailed analysis of the best two media (i.e. KB and CAA) showed that pyochelin production is less hampered in KB medium than pyoverdine (with a relative production of about 50 and 100% of CAA respectively) (Figure 10). This observation suggests that *Pseudomonas* sp. CMR12a endures a strong iron deficiency upon growth in CAA medium but only experiences a moderate iron stress while grown in KB medium. Indeed, pyochelin is produced at first, when the iron stress is moderate and pyoverdine synthesis is triggered when the need for iron is stronger (Drehe et al., 2018).

3.2.2. The test of mutants revealed that pyoverdine is involved in the boost unlike the antimicrobials sessilin, orfamide and phenazine.

In order to more specifically discriminate the involvement of the known BSM, we tested a range of mutants impaired in the synthesis of these metabolites (figure 11). Data showed that none of the mutants unable to produce one, two or all three of the antimicrobial compounds (i.e. sessilin, orfamide and phenazine) had lost their ability to induce an increase of dihydrobacillaene production.

The only mutant that lost most of its activity is the *Pseudomonas* sp. CMR12a 2.95 mutant unable to synthesize pyoverdine. This further supports the involvement of this siderophore in triggering the *Bacillus* response.

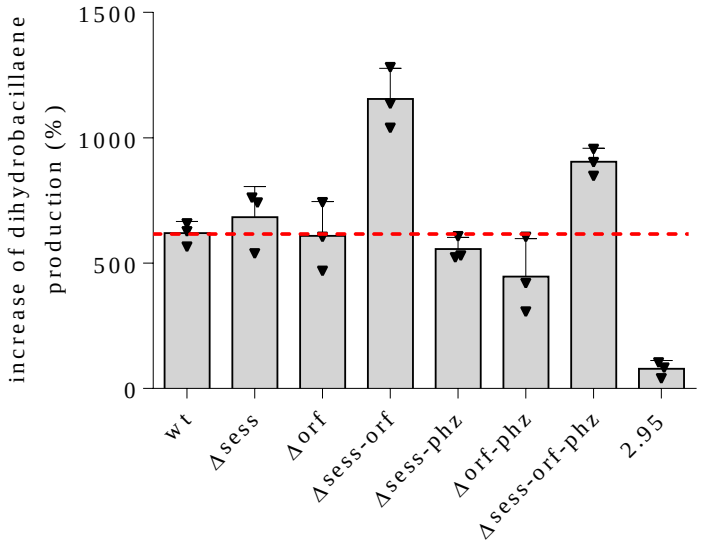


Figure 11: ***Evaluation of the triggering activity of the supernatant of*** **Pseudomonas** ***sp. CMR12a mutants.*** Bacillus velezensis *GA1 cultures were supplemented with 2% (v/v) of various mutants of* Pseudomonas *sp. CMR12a.. Δsess: mutant lacking the ability to produce sessilin, Δorf: mutant lacking the ability to produce orfamide, Δphz: mutant lacking the ability to produce phenazine. 2.95: mutant lacking the ability to produce pyoverdine.*

As none of the antimicrobials are involved in the triggering activity, as the CLPs are toxic to the *Bacillus* cells and as the double mutant *P.* sp. CMR12a Δsess-orf was the most efficient, it was decided to use this mutant for the following experiments.

3.2.3. The iron deficiency induced by the siderophores pyoverdine and pyochelin boost the production of dihydrobacillaene

The supernatant of *Pseudomonas* sp. CMR12a grown in CAA and iron supplemented CAA were fractioned on C18 cartridges and eluted with increasing acetonitrile/water ratio. The siderophores' (pyoverdine and pyochelin) contents of each fraction collected were evaluated and the triggering activities of these fractions were assessed (Figure 12).

We notice a correlation between the siderophore content and the increase in dihydrobacillaene production. Interestingly, fractions containing only pyoverdine or pyochelin were effective, suggesting the implication of siderophores in the boost is mediated by their iron scavenging activity. Moreover, the most efficient fractions are fractions 15% and 20% ACN, which corresponding to be the fractions containing both siderophores. This observation suggests an additive effect of those two molecules.

However, a residual boosting effect is observed for the fraction 20% ACN collected from the iron supplemented CAA medium. This observation, in link with the OSMAC experiment (Figure 10) where iron supplementation did not cause a total loss of activity illustrates that at least another trigger is present (Figure12).

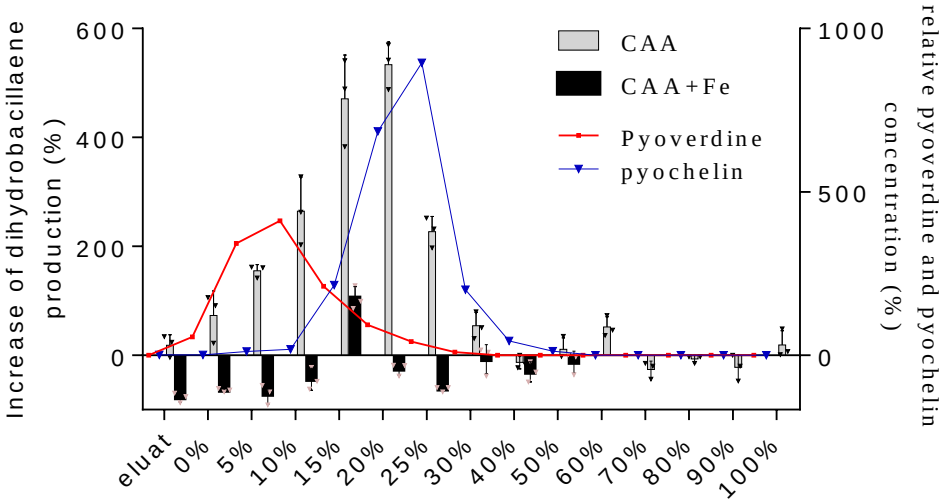


Figure 12: ***Triggering activity of the fractions of*** **Pseudomonas** ***sp. CMR12a supernatant in relation to their siderophore content****. The fractions correspond to successive elutions with solutions of growing ACN-water ratio expressed in %(v/v) of ACN.* Bacillus velezensis *GA1 cultures were supplemented with 4% (v/v) of the fractions of* Pseudomonas *sp. CMR12a.Δsess-orf supernatant grown either in CAA and CAA+Fe medium. The siderophore content was measured for the supernatant generated in CAA medium. The siderophore content is expressed in % of the crude sample.*

In order to further demonstrate the implication of the pyoverdine and pyochelin in the triggering activity via the induction of an iron stress we used purified siderophores to prove their triggering

activity. Detailed information of the purification process is available in section 4. of the annex. The concentrations of the purified compound used in this experiment correspond to the concentrations in the crude sample *Pseudomonas* supernatant. Using the same method of pic areas ratio as for the relative quantification of pyoverdine and pyochelin in the OSMAC experiment, an appropriate volume of the purified molecule was determined to fit the concentration of the *P.* sp. CMR12a supernatant.

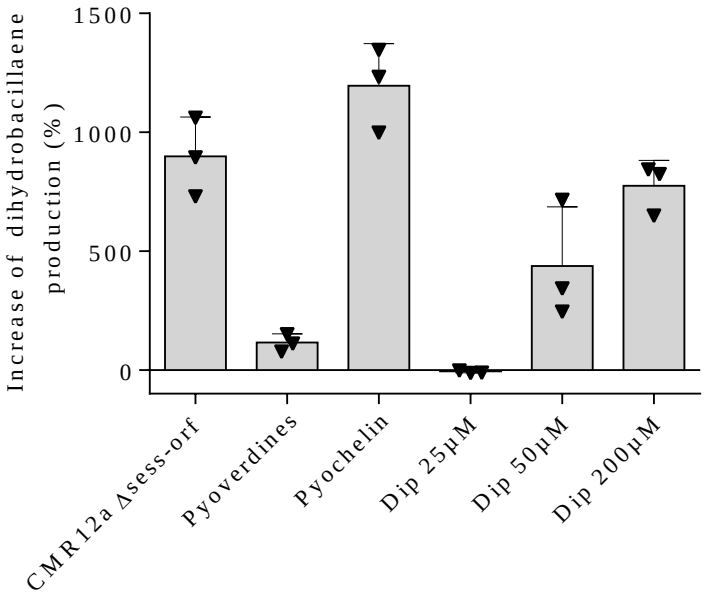


Figure 13: ***Triggering effect of iron chelatant. on the dihyrobaillaene production of*** **Bacillus velezensis *GA1*.** Bacillus velezensis *GA1cultures were supplemented with 4% (v/v) of* Pseudomonas *sp. CMR12a Δsess-orf, pure pyoverdines, pure pyochelin or growing concentrations of Dip (25, 50 and 200µM).The concentration of pyoverdines and pyochelin are equivalent to 4% (v/v) of* Pseudomonas *sp. CMR12a Δsess-orf superantant.*

According to Figure 13, this experiment shows that, even though pyochelin is more efficient than pyoverdine at those concentrations, both siderophores are able to induce an increase in dihydrobacillaene production. It also demonstrates that the DIP induces a boost as well. The DIP is a structurally unrelated molecule that only has in common with the siderophores the ability to chelate iron and thus induce an iron stress. As a consequence, we postulate that the antimicrobial overproduction is not due to the ability of *Bacillus* to recognize a molecule or a molecular pattern associated with the siderophores but rather that *Bacillus* responds to sensing some iron deficiency. This idea is further supported by the fact that both the *Pseudomonas crude* supernatant and the DIP(at 200µM) induce a sharp boost of bacillibactin, the *B. velezensis* GA1 siderophore, (an increase of 1100% and 750% respectively whereas the supernatant of the iron supplemented culture, which contained no siderophores does not enhance the bacillibactin production (Figure 32,section 5 on the annex)).

Next, the increase in DIP concentration induces an increase in the magnitude of the antimicrobials production, and thus a dose response relationship can be considered.

Finally, the DIP concentrations from 25 to 200µM are subinhibitory as no OD differences were noticed while higher concentrations (400 and 600µM) inhibited the growth of *Bacillus* (Figure 33,section 6 on the annexes). We can thus expect the presence of an iron-stress optimum for BSM triggering followed by a growth inhibition for higher iron shortages.

Taken together, these results indicate that the *Bacillus* cells perceive an iron stress created by *Pseudomonas* sp. CMR12

Finally**,** in order to prove the involvement of siderophore in the triggering activity of *B. velezensis* GA1, we conducted a chemical complementation assay of *Pseudomonas* sp. CMR12a 2.95 (unable to produce pyoverdine) with pure pyoverdine and pyochelin (Figure 14).

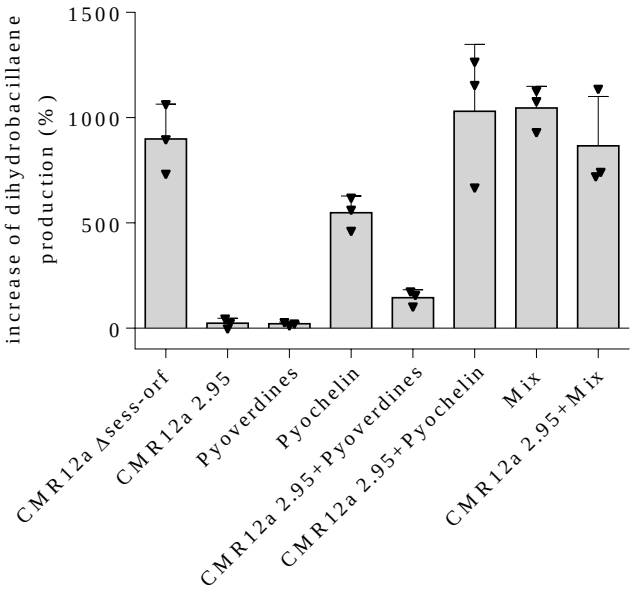


*Figure 14:**Chemical complementation of* **Pseudomonas** ***sp. CMR12a 2.95 with siderophores renders its aptitude to boost the production of antimicrobials of*** **Bacillus velezensis** ***GA1****. 1%(v/v) of Pseudomonas supernatant was added to the* Bacillus velezensis *GA1 culture or the amount of pure pyoverdine, pyochelin or the mix of the two corresponding to 1%(v/v)of* Pseudomona*s sp.CMR12a Δsess-orf supernatant*

The results show that, as expected, *Pseudomonas sp.* CMR12a 2.95 lost almost all of its triggering activity. however, complementation with pyoverdine alone allowed to recover only a small part of the effect while addition of pyochelin allowed to almost fully restore the triggering potential of the extract. Yet the complementation of the mutant 2.95 with both pyoverdine and pyochelin yielded a similar effect on the PKS triggering activity. This leads to

the conclusion that both siderophores are able to induce an iron deficiency sufficient to trigger the production of PKs by *Bacillus*.

We can now assert that *Pseudomonas* sp. CMR12a siderophores induce an iron deficiency that is perceived by *Bacillus velezensis* GA1 and that, in response to the stress, *Bacillus* increases its production of PKs such as dihydrobacillaene.

3.3. Different strains of *Pseudomonas* are able to induce an iron deficiency related boost

To further investigate the specificity of PKS stimulation in *B. velezensis* GA1by *Pseudomonas* extracts, we examined the effect of crude extracts prepared from other strains isolated from soils belonging to the *Pseudomonas* generus (Figure 15 and Table8). In order to do so, various strains were tested upon their triggering activity (Figure 15). The *Pseudomonas* sp. CMR12a, COW8, COR58 and COR33 strains are from cocoyam roots. They are not yet assigned to a species. (COW: white cocoyam. COR: Red cocoyam) (Perneel et al., 2007; Oni, Geudens, et al., 2019), while CH36 belongs to the species *P. tolaassii*, and Pf-5 to *P. protegens*.

Table 8: ***Diversity of putative siderophore produce by Pseudomonas strains****. In the column 'Pyochelin' the presence of the metabolite is marked with a '+' and the absence with a '-'.*

Strains	**Pyochelin**	**Pyoverdines**
		Monocharged main ions (m/z)
CMR12a	+	1306.5606 and 1335.6159
PF-5	+	1306.5606 and 1335.6159
COW8	-	1322.6344 and various minor pics
COR58	-	-
COR33	-	1222.5686 and 1257.4365
CH36	-	1425.6242, 1442.6760 and 1454.6179

Interestingly, only the two closely related strains *Pseudomonas* sp. CMR12a and *Pseudomonas protegens* PF-5 produce pyochelin and COR58 is the only strain that does not produce any detectable pyoverdine. Except *P.* sp. CRM12a and *P.protegens* PF-5, all strains produce a structurally specific pyoverdine based on the exact mass of the molecular ion in LC-MS (Table 8)

The triggering activities of the corresponding crude extracts were assessed upon growth of the producing strain both in CAA and in iron supplemented CAA medium. Interestingly, a boost

in PKs synthesis has been observed for every strain when grown in CAA medium. Moreover, each strain but *P.* sp. COR58 had a higher boost when grown in CAA compare to in iron supplemented CAA medium. Interestingly, *P.* sp. COR58 is the only strain that did not produce any of the two siderophores. This observation reinforces the hypothesis of iron stress as a trigger.

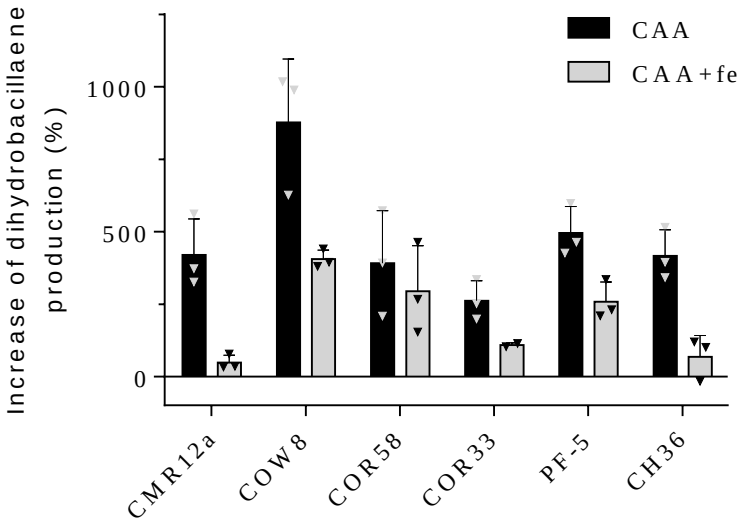


Figure 15: ***Triggering activity of various*** **Pseudomonas** ***strains****. The increase of dihydrobacillaene production is calculated as the increase of pic area of dihydrobacillaene (% of the control group).* Bacillus velezensis *GA1 culture supplemented with with 4% (v/v) of* Pseudomonas *sp.*

Nevertheless, data in Figure 15 also show that extracts from most strains had a triggering activity upon growth in iron-supplemented medium, supporting the hypothesis that there is another trigger that remains to be discovered.

This experiment also shows that the pyoverdine produced by *Pseudomonas spp*. induce an iron deficiency sufficient to trigger a boost, independently of the structure and thus affinity constant of the siderophore.

Chapter 4: Discussion and perspectives

1. *Pseudomonas* sp. CMR12a secondary metabolome

This work first focused on the chrarcterization of *Pseudomonas* sp. CMR12a BSM as it was a *sine qua non* condition to properly study the bacterial interaction.

The gap between the variety of BSM predicted by genome mining with antiSMASH 5.0 and those detected in our UPLC-qTOF MS analyses suggests that there are many cryptic genes yet to be awoken. The use of more stressful conditions or conditions closer to the natural context (nutritional context best mimicking root exudates, interspecies competitive interactions with other rhizobacteria or other competitive soil bacterial species, cultures as biofilms on solid medium, etc…) may be the key to get a significant transcriptional level of those genes and detect their products in the culture supernatant.

The elucidation of the structure of the CMR12a pyoverdines revealed that *Pseudomonas* sp. CMR12a produces two slightly different variants. Interestingly, these pyoverdines are structurally similar to the one described for the closely related *Pseudomonas* Pf-5 (Hartney et al., 2013). Along with the structure, these authors have also described the genetic cluster of the *P. protegens* Pf-5 pyoverdine which is very similar to one of the two clusters found by antiSMASH for the synthesis of pyoverdines in *P.* sp. CMR12a (Figure 4).

The elucidation of the structure of the second variant remains to be performed based on the exact mass with tools such as NMR and MS-MS,. Moreover, it would be interesting to investigate whether each cluster codes for the synthesis of a variant of pyoverdine or whether the two variants come from an unspecific enzyme encoded in one of the biosynthetic clusters.

2. Interplay between CLPs drives the antagonistic interaction between *Pseudomonas* sp. CMR12a and *Bacillus velezensis* GA1

We investigated the interplay between surfactin and sessilin. The toxicity of sessilin and the protective role of surfactin illustrate the complex role of these metabolites and new functions for lipopeptides in general. This adds to the multifunctionality of these molecules as sessilin is also involved in biofilm and swarming regulation (D'aes et al., 2014) in addition to its antifungal properties (Oni et al., 2019) while surfactin is well-known for its plant immunity elicitor activity, and its involvement in biofilm formation and motility regulation (Ongena et al., 2008).

The results add up to the general knowledge of the *Bacillus* behavior upon interspecies interactions or cross-talk. Several mechanisms developed by *Bacillus* to avoid antagonistic interactions have recently been reported. First, the hydrophobic biofilm produced by *Bacillus subtilis* was described as a physical barrier protecting from *Pseudomonas* (Molina-santiago et al., 2019). Second, the induction of the sporulation mechanisms was also observed that can be driven either by a decrease of nutrient availability or an environmental change due to the presence of other bacteria or by the perception of a molecule (probably T6SS) (Molina-santiago et al., 2019). Finally, it was showed that the overproduction of surfactin in presence of other colonies promote the bacterial spread (Molina-santiago et al., 2019). The results of our work add up to the palette of avoidance strategies as surfactin could act as a chemical barrier toward sessilin and reacts with it to neutralize its toxicity via a kind of co-precipitation visualized as the formation of a white line in the interaction zone between the two colonies on gelified medium (Figure 7). Despite the strong indications we obtained in this work, conclusive evidence for the antibacterial activity of sessilin toward *B. velezensis* GA1 would require an experiment with chemical complementation of the *P.* sp. CMR12a Δsess mutant with pure sessilin or a genetic complementation via the introduction of a plasmid containing the deleted gene to restore its function.

In a broader perspective, the white line formation as a defense mechanism should be investigated more deeply. The potential of surfactin and sessilin could be screened upon various lipopeptides and other antimicrobials. To study whether the surfactin offers a protection toward a wide variety of exogenous CLPs would offer a better understanding of the complex role of

this metabolite contributing to the fitness of *B. velezensis*. Moreover, the discovery of such a phenomenon upon various CLPs of various bacterial strains and species along with the evaluation of its implication on the bacterium fitness would offer a better understanding of the microbial antagonism and avoidance strategies.

From an ecological point of view, the role of surfactin and sessilin, with regard to their molecular interaction, should be investigated *in planta* upon dual root colonization of *P. sp.* CMR12a and *B. velezensis* GA1. Preliminary work done in the lab shows that unwashed *Bacillus* cells have a better colonization and survival ability while in contact with sessilin producing *Pseudomonas*. These observations suggest that the BSMs of *Bacillus* improve its fitness and helps it resists *Pseudomonas* pressure. The co-inoculation of sessilin and/or surfactin mutants would help decipher whether it is the WLIP phenomenon that drives the improved fitness observed.

3. Iron deficiency mediated trigger

The third but most important part of the work focused on the stimulation of antibacterial polyketides by *B. velezensis* in response to *Pseudomonas*. Our results indicate that the siderophores produced by *P.* sp. CMR12a play a major but indirect role by inducing some iron scarcity rather than being recognized as a molecular signal *per se*. Such iron deficiency is perceived by *B. velezensis* GA1 and induces not only the production of bacillibactin (the GA1 siderophore) but also an increase in the production of antimicrobial PKs. Our work focused on dihydrobacillaene production. Dihydrobacillaene was originally described as a broad-spectrum antibacterial compound that interferes with protein synthesis in gram negative target cells (Patel et al., 1995). It is also known to protect *Bacillus* from predation as it slows down the growth of the predator *Myxococcus xanthus* which spares enough time for *Bacillus* to form spores that are resistant to predation (Müller et al., 2014). We used dihydrobacillaene production as a proxi but the iron-stress dependent interaction with *Pseudomonas* has an impact on other BSMs. Indeed, additional results from the MiPI lab suggest that amylocyclycin, a cyclic bacteriocins with anti-Gram-positive activity (Scholz et al., 2014), is overexpressed too. These observations strongly suggest that this BSM is the main driver of the anti-*Clavibacter* activity. Interestingly, this strategy is resolutely aggressive and proactive, unlike the other strategies mentioned above (i.e. sporulation and chemical and physical barrier). Indeed, in this case, the bacterium reacts by fighting whereas the other behaviors aimed to avoid contact and to protect from the outside aggressions or stresses.

The effect of iron limitation provoked by a competitor on antimicrobial biosynthesis regulation in *Bacillus* illustrates a novel facet of interspecies outcomes. The BSM production may be finely regulated (Figure 16). But, to our knowledge, no iron-related regulators have been described to impact the production of PKs and especially difficidin and bacillaene (Figure 16). So far, few iron-dependent regulation mechanisms have been reported for *Bacillus*. It includes the Fur (Pi et al., 2017) and Per systems (Baichoo et al., 2002) (Figure 16). However, these systems are only known to regulate iron-scavenging related genes such as those coding for the synthesis of siderophores and associated ferri-siderophore membrane transporters. Thus, the link between iron and the regulation of antimicrobials in *Bacillus* remains unknown. An important perspective would be to study the biomolecular mechanism underlying this phenomenon. An *in silico* analysis of the promotor-regulator affinity should be considered first to predict whether an iron regulator can bind to the promoter region of antimicrobial

biosynthetic gene clusters. Another approach would be to test the regulators of the expression of antimicrobials and test their sensitivity to iron homeostasis. A simple way to proceed would be to knock out the regulator and compare the antimicrobial production in different iron stress conditions.

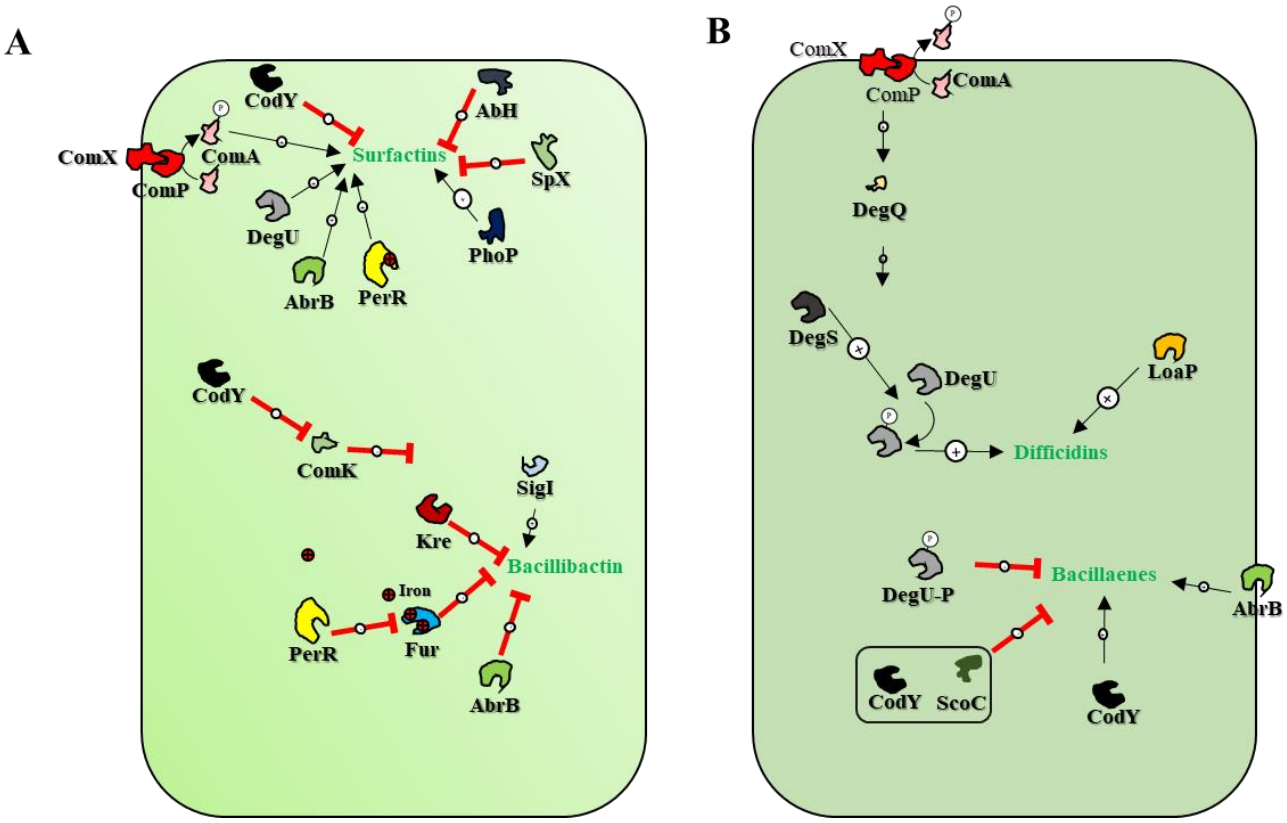


Figure 16: **Bacillus *BSM regulation.*** *From S. Andric et al., unpublished. The black arrows represent an activation while the red bars represent a repression of the gene expression. The red crossed circles (⊕) represent iron atoms, the promotors with the symbol are iron regulated (i.e. Fur and PerP). The circled 'P' represents a phosphorylation. Figure 16.A: NRPSs regulation. Figure 16.B. PKSs regulation.*

Considering the *'Pseudomonas* side of the interaction', the results open a door to a better understanding of the role of siderophores. Indeed, so far, the impact of siderophores in plant health improvement has been limited to its ability to make iron available for the PGPRs and the plant, to its bacteriostatic activity and to its role in the competition for ferric ions (Bertrand et al., 2014; Goswami et al., 2016). Our results suggest that it may also retain an indirect role of enhancing the antagonist potential of PGPRs. Nevertheless, in *Bacillus*, the role of siderophores is quite complex. *Bacillus* is able to utilize xenosiderophores thanks to the FeuA transporter that imports the metabolite inside the cell and the hydrolase ybbA which releases the iron by degrading the ferri-siderophore (Grandchamp et al., 2017). This extra iron may trigger sporulation. The authors suggest that the extra iron binds to the PerR regulator which positively

regulates the *srfA* operon of surfactin synthesis (Figure 16),the latter being involved in sporulation (Grandchamp et al., 2017). However, our results suggest that the mechanisms underpinning antimicrobial overproduction are not linked to an excessive iron concentration but rather to an iron deficiency. Indeed, medium supplementation with the DIP iron chelator resulted in a boost of PKs production but *Bacillus* is not able to incorporate the DIP-Fe complex (Grandchamp et al., 2017). These two observations suggest a dual role of iron homeostasis in the ability of *Bacillus* to detect the presence of other bacteria and in the response *Bacillus* will adopt. It would be interesting to investigate whether the iron deficiency has an impact on the physiology of the cells as well as the iron over abundance (i.e. compare with precision the growth rate, the production rate of the different BSM over time, the cells physiology, the dynamic of sporulation rate…)

In addition to iron deficiency, the presence of another *Pseudomonas* compound, non-iron related, but retaining PKS-triggering activity is highly suspected. We showed that this trigger is produced by various *Pseudomonas* strains upon growth in CAA medium (both with and without iron). Thus, we can argue that this trigger is well conserved among the *Pseudomonas* genus and could therefore be essential for *Pseudomonas* fitness. The *Bacillus* ability to detect such conserved molecules would be powerful tool to detect the presence of surrounding *Pseudomonas*. Moreover, The discovery of an additional interaction system would, of course, add up to the complexity of the interaction studied. In order to investigate this trigger, we have to keep in mind that KB and CAA media were the two media in which CMR12a produced a sufficient amount of trigger molecules. Further, the iron supplementation of the CAA medium did not lead to a total loss of the triggering activity. However, the content of siderophore was lower on the KB medium in comparison to the CAA medium. So, we can hypothesize that the supernatant grown in KB medium contains a decent amount of the non-iron related trigger that compensates the lower siderophore content. Thus, it would be interesting to fractionate KB and iron-supplemented KB crude supernatant as it was done for CAA and compare the active fractions in terms of metabolite content and PKS stimulating activity in parallel.

Next, It has already been reported that the production of secondary metabolites in *Bacillus* is boosted when bacterial growth is limited by C, N P or other nutrient shortage (Tyc et al., 2016). When considering not specifically iron but nutrient depletion as a mechanism of recognition of the presence of neighboring microorganisms, the question that arises is whether the starvation of other nutrients can also be perceived by *Bacillus* and whether this perception can induce a similar proactive response. Multiple assays can be run with root exudates medium depleted with

one or several essential nutrients to mimic the impact of various nutrients uptake by the surrounding microbial community. The pyochelin mutant and double mutant pyoverdine and pyochelin could also be further tested for their ability to trigger antimicrobial overproduction. Moreover, this experiment could provide some clues to understand why pyochelin has such a great inducing activity in comparison to pyoverdine. This question could also be answered with the quantification of the iron deficiency needed to induce the PKs overproduction by *Bcillus* in correlation with a precise quantification of pyoverdine and pyochelin content.

As for the ability of various *Pseudomonas* to induce a boost of PKs production on *B. velezensis* GA1, it would be interesting to test the effect of *Pseudomonas-Bacillus* interaction on various Bacilli root-associated or not, to see whether the perception of iron deficiency (and possibly another non-iron-related trigger) and response by overproducing PKs is a wide spread ability among Bacilli and whether it is specific to root-associated Bacilli. If the second hypothesis appears to be true, it would open a way to the investigation of it being an evolutionary traits selected for the rhizosphere ecological niche.

Finally, the transposition of the lab conditions to in planta experiment remains to be done. Attempts were made to cultivate *Bacillus velezensis* GA1 and *Pseudomonas* sp. CMR12a on tomato roots in the MiPI lab. However, so far, in the conditions used, *Pseudomonas* overgrew *Bacillus* in a way that the *Bacillus* cells were practically undetectable after a couple of days. Thus no boost of PKS could be confirmed. The validation, in planta, of the results here obtained is a crucial step to support the ecological relevance of the mechanisms discovered in this work.

4. General conclusion

The outcome of this work lines up with research in the scope of a larger and more global context. In spite of the limited allotted time for the experiment, it hopefully provides some new knowledge to the understanding of how root-associated *Bacillus* species may behave, interact and communicate with other bacteria sharing their natural environment. A better understanding of the molecular interactions among rhizobacteria and, more generally, of the mechanisms underlying the dynamics of the microbial communities in the rhizosphere, is a promising step forward toward a better understanding of the factors and mechanisms influencing the outcome of biocontrol agents and their efficiency. In that sense, it could contributes, though modestly, to the necessary effort of the scientific community to improve the way to use these PGPR isolates for optimal biological control of plant diseases and thus for the improvement of more sustainable cropping practices. The perspectives it opens encourage to invest with enthusiasm and engagement in further research in the field.

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Annex

1. Background

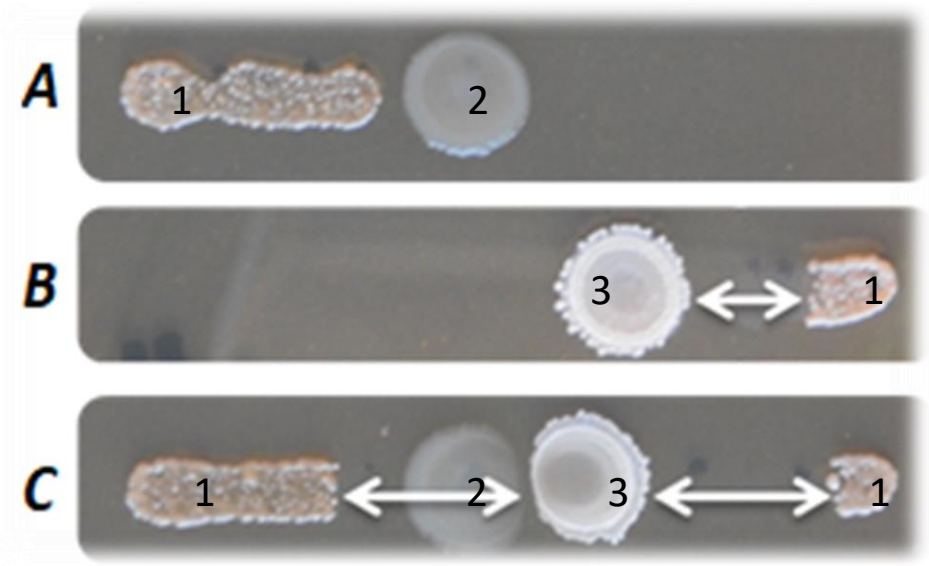

Figure 17: ***Solid interaction between*** **Clavibacter michiganensis** ***subsp.*** **michiganensis** ***(1),*** **Pseudomonas** ***sp. CMR12A (2) and*** **Bacillus velezensis** ***GA1(3)*** *(Sofija Andric, unpublished). Figure 18.A : interaction between* C. michiganensis *and* Pseudomonas *sp. CMR12A Figure 18.B: interaction between* C.michiganensis *and* B. velezensis GA1. *Figure 18.C: interaction between* C. michiganensis, Pseudomonas *sp. CMR12a and* B. velezensis GA1. *The white arrow represents the inhibition zone of C. michiganensis.*

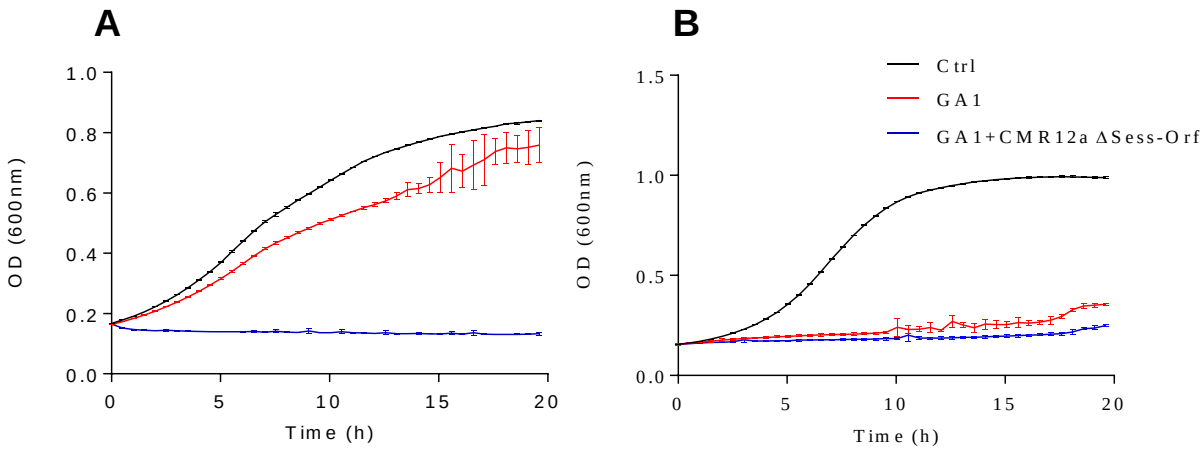

Figure 18: **Pseudomonas** ***sp. CMR12a Δsess-orf supernatant boost of the antimicrobial activity of*** **Bacillus velezensis** ***GA1.*** *A: toward* Clavibacter michiganensis *and B. toward* Xanthomonas campestris *pb.* campestris. *Ctrl: pure* Clavibacter *and Xanthomonas culture; GA1:* C. michiganenesis *and* X. campestris *cultures with 1% and 6% of* Bacillus velezensis *GA1 supernatant respectively; GA1+CMR12a Sess-Orf:* C. michiganensis *and* X. campestris *culture with 1% and 6% of* Bacillus velezensis *GA1 supernatant primed with 4% of* Pseudomonas *sp. CMR12a Δsess-orf mutant supernatant respectively.*

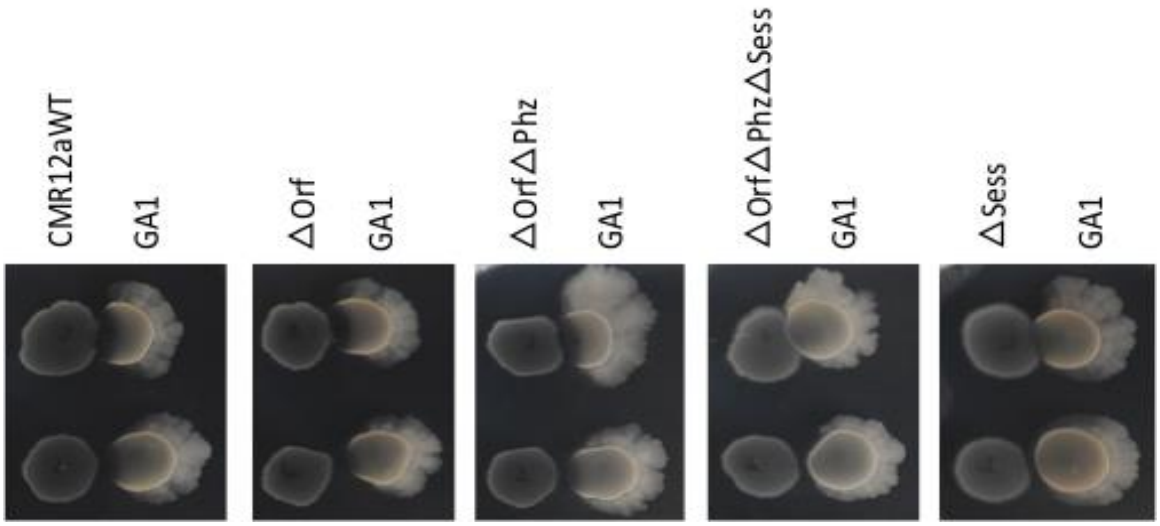

Figure 19: **Bacillus velezensis *GA1 and* Pseudomonas *sp. CMR12a mutants solid interaction***.

2. pyoverdine identification supplement

Both *P.* sp. CMR12a and *P. protegens*PF-5 have two variants of pyoverdine: one with a m/z of 1288.5800 ratio for the main ion (or 1306.5906 when hydrated) (in red) and one with a m/z ratio of 1335.6159 (in green) (figure 20). The retention times are 7.5 and 7.4 min respectively. Figure 20.C illustrates the pyoverdines distribution of *P.* sp. CMR12a. The exact similar observation is noticed for *P. protegens* PF-5 (Figure 20.B).

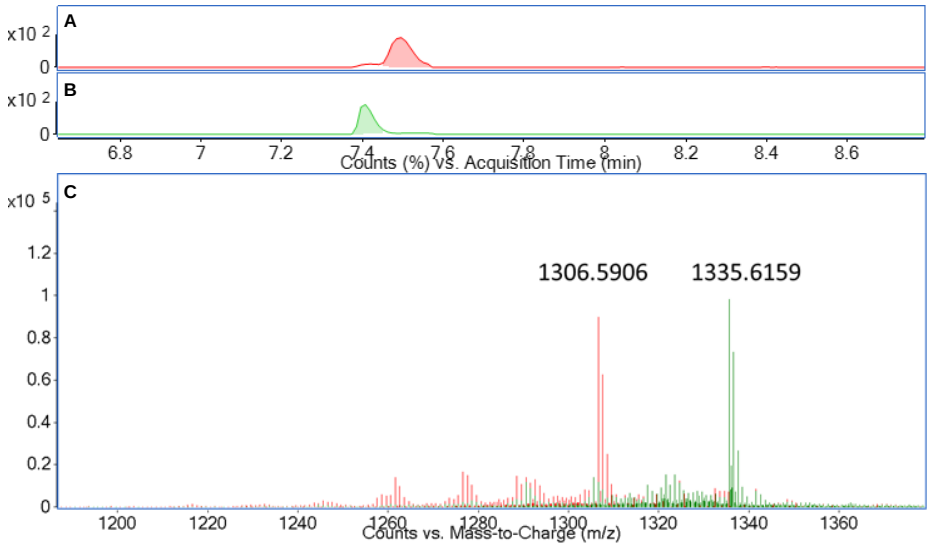

Figure 20: ***Chromatograms and mass spectrum of the* Pseudomonas *sp. CMR12a pyoverdines***. *A(in red):chromatogram of the ion m/z=1288.58, (Rt of the pic=7.462-7.540 mn); B(in green):chromatogram of the ion m/z=1335.61, (Rt of the pic=7.385-7.440mn) C:Mass distribution of the two pyoverdine pics. In red: pic with Rt=7.462-7.540 mn; in green pic with Rt=7.385-7.440mn*

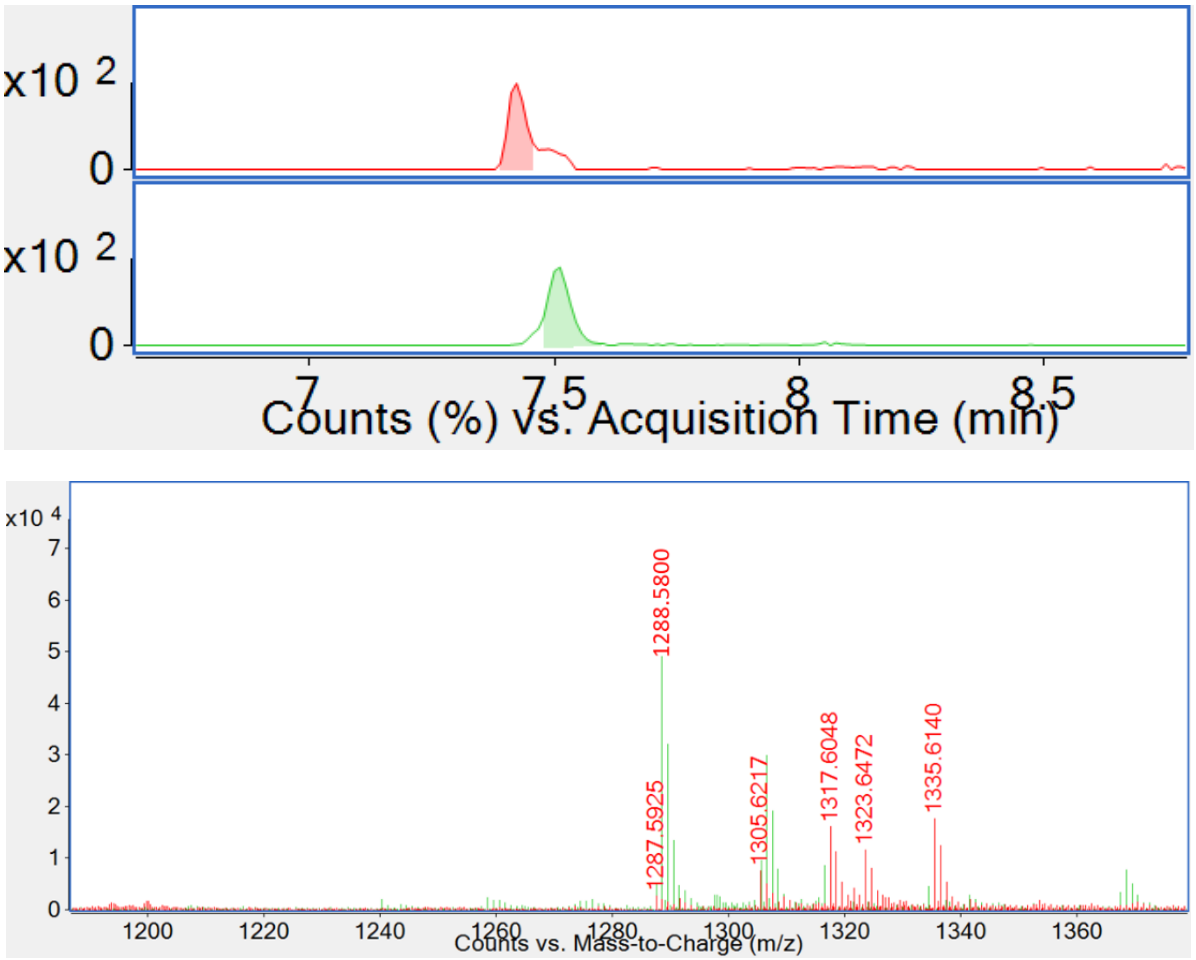

Figure 21: ***Chromatogram (at the top) and mass spectrum (at the bottom) of the pyoverdines of*** **Pseudomonas protegens *Pf-5*.** *In green, m/z of the most abundant ion 1255.5800. In red, m/z of the most abundant ion= 1335.6140*

To further compare the pyoverdines of *P.* sp. CMR12a and *P. protegens* PF-5, the mass distributions and UV-Vis spectra are overlaid (figure 22 and 23). One can notice that the ions have the same exact mass (figure 22). The only change is the relative abundance of each "bloc"[5] of ions, which suggests that in the *P.* sp. CMR12a sample, the hydrated form is more abundant, whereas for *P. protegens* PF-5, the non-hydrated form is the most abundant. The ion 1288.5800 was assigned as the non-hydrated ion as it fits the mass of the pyoverdine of *P. protegens* PF-5 described by (Hartney et al., 2013). The DAD spectra (figure 23) further support the idea that the two strains produce the same pyoverdines as their DAD spectra are highly similar. Moreover, the retention times are essentially the same for both variants of *P.* sp. CMR12a and *P. protegens* PF-5 (table 9).

[5] The « bloc » here refers to the isotope distribution of a molecule. The fact that the ion 1288.5800 and 1306.5914 have the same isotope distribution is in line with the idea of a single molecule with a hydrated form.

*Table 9:**Rentention times of the main monocharges ions of* **Pseudomonas protegens** ***PF-5 and*** **Pseudomonas** ***sp. CMR12a pyoverdines***

m/z	Rt CMR12a	Rt Pf-5
1288.5813	7.517(-7.65-7.727)	7.511
1306.6094	7.495	7.489
1335.6082	7.407	7.423

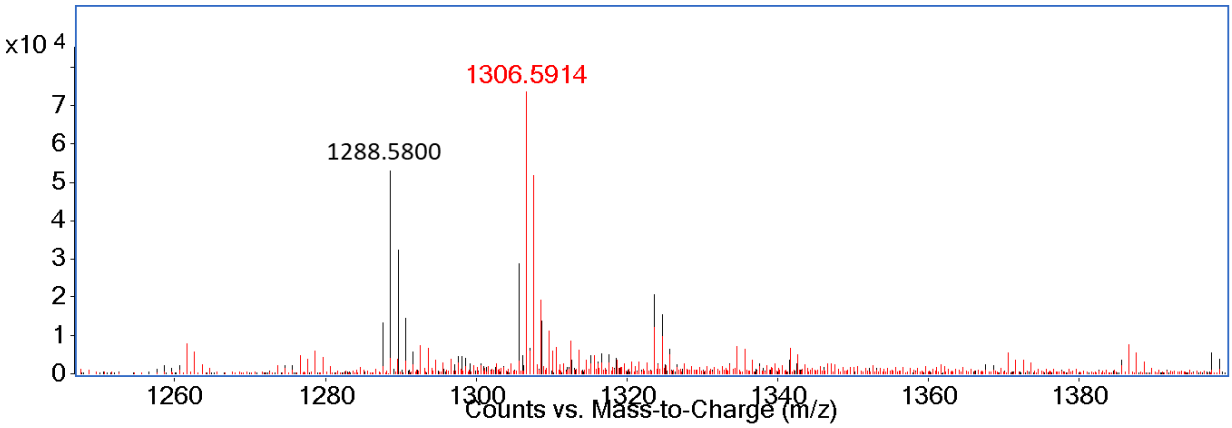

*Figure 22: **Mass spectrum of*** **Pseudomonas protegens** ***PF-5*** *(in black)* ***and*** **Pseudomonas** ***sp.CMR12a*** *(in red) pyoverdines*

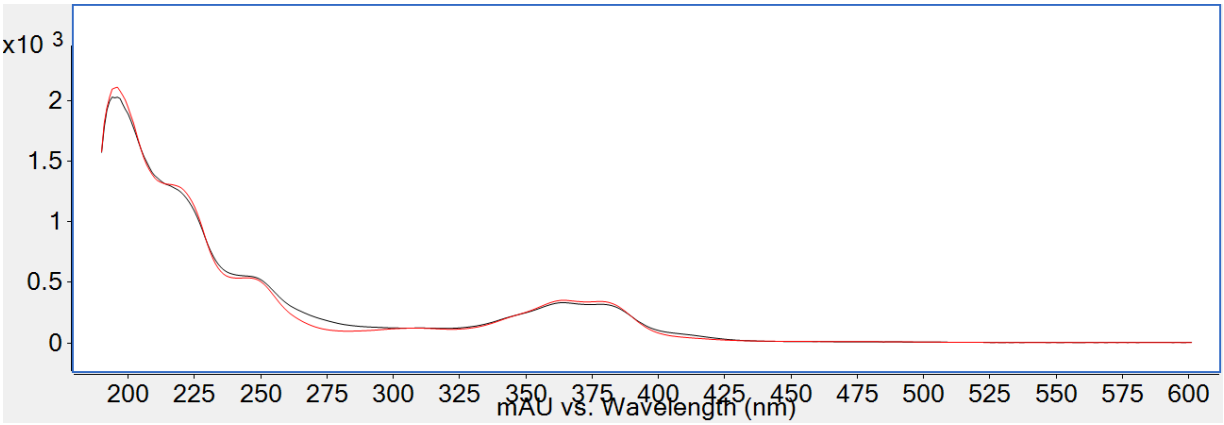

*Figure 23: **UV-Vis spectrum of*** **Pseudomonas protegens** ***PF-5*** *(in black)* ***and*** **Pseudomonas** ***sp. CMR12a*** *(in red)* ***pyoverdines***

3. The iron supplementation inhibits both the pyoverdine and pyochelin production

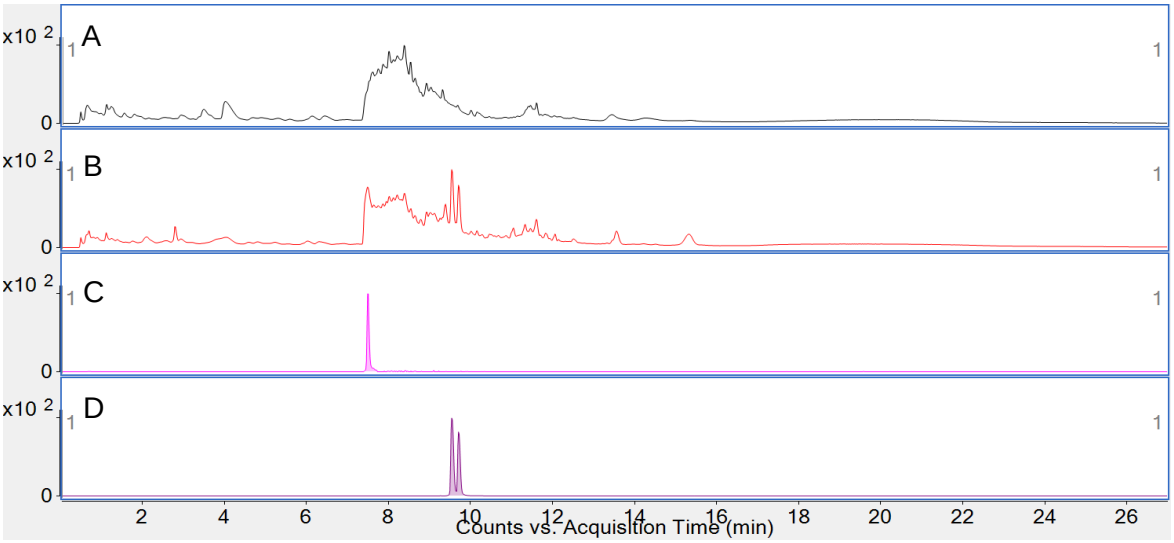

*Figure 24**: UPLC-MS chromatogram of* **Pseudomonas** ***sp. CMR12a supernatants****. A: TIC of iron supplemented CAA medium. B:TIC of CAA medium. C:EIC (m/z=1306.5900) (pyoverdine)of CAA medium D. EIC (m/z=325.0690) (pyochelin) of CAA medium*

4. Siderophores purification

The purification of the siderophores was performed in two steps: the semi-purification of the siderophores and the concentration of them on a C18 cartridge followed by the a purification step by HPLC.

As mentioned, the *P.* sp. CMR12a supernatant was loaded on a C18 cartridge. A first fraction was collected with a solution of water-ACN 85-15% (v/v). This fraction was enriched in pyoverdine. Figure 26 illustrates the overlaid chromatogram of the absorbance at 320 and 380 nm[6]. The second fraction was collected with a solution water-ACN 70-30% (v/v).Figure 27 illustrates the overlaid chromatogram of the absorbance at 320 and 380nm.

[6] 320nm corresponds to the maximal absorbance of pyochelin and 380nm corresponds to the maximal absorbance of pyoverdines. The two wavelengths were used to discriminate one molecule from the other and make sure they were present in the enriched fraction

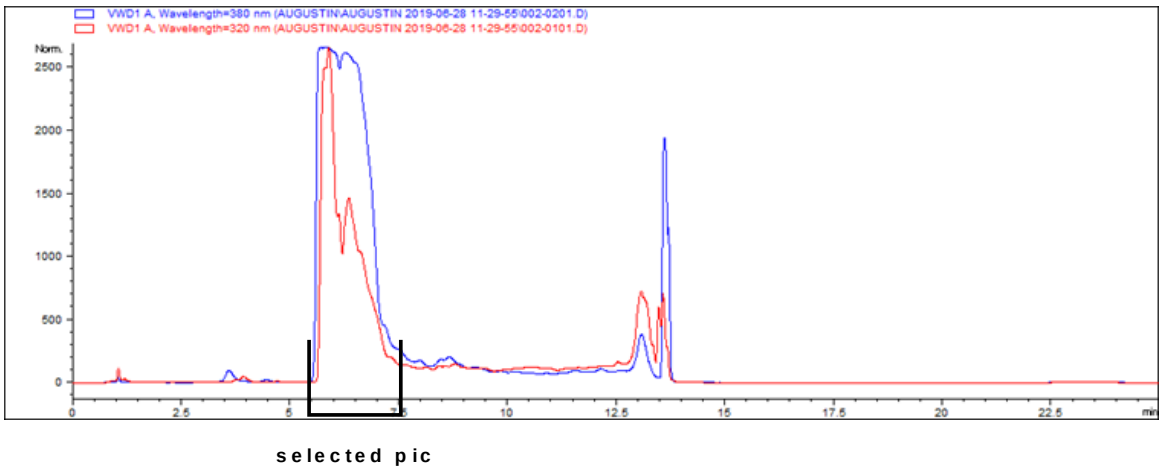

*Figure 25:**Chromatogram of the prepurified pyoverdine**. In red: absorbance at 320 nm. In blue: absorbance at 380nm*

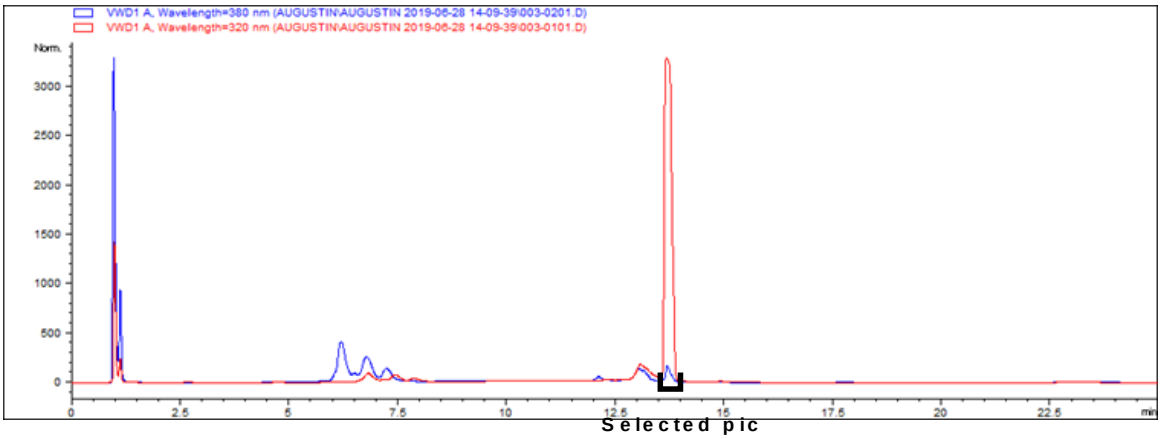

*Figure 26:**chromatogram of the prepurifed pyochelin**. In red: absorbance at 320 nm. In Blue: absorbance at 380nm*

Next, the enriched fractions were injected in HPLC and the eluate was collected at the time corresponding to the pyoverdine and pyochelin (figure 26 and 27 respectively).

Once the eluates were collected, the presence of pyoverdine and pyochelin in the samples were confirmed with the UV-Vis spectrum and the mass spectrum of the molecule (figure 28,29,30,31).

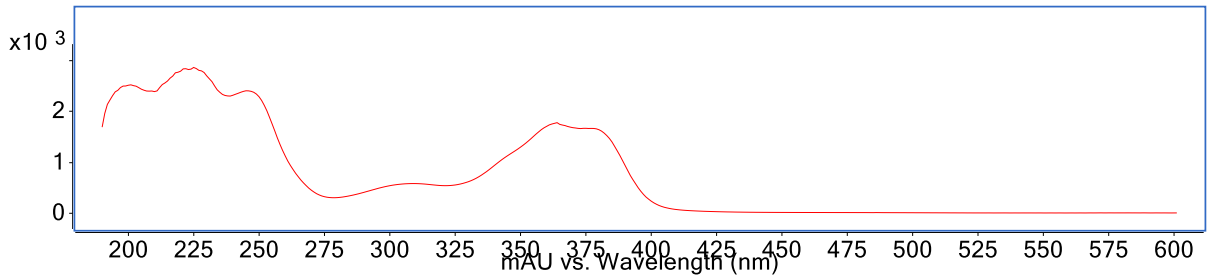

*Figure 27: **UV-vis spectrum of purified pyoverdine***

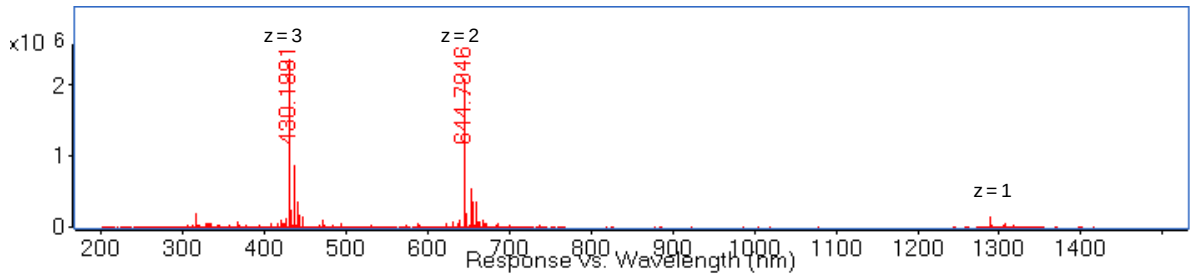

Figure 28: ***Mass spectrum of the purified pyoverdine****. z : number of charges*

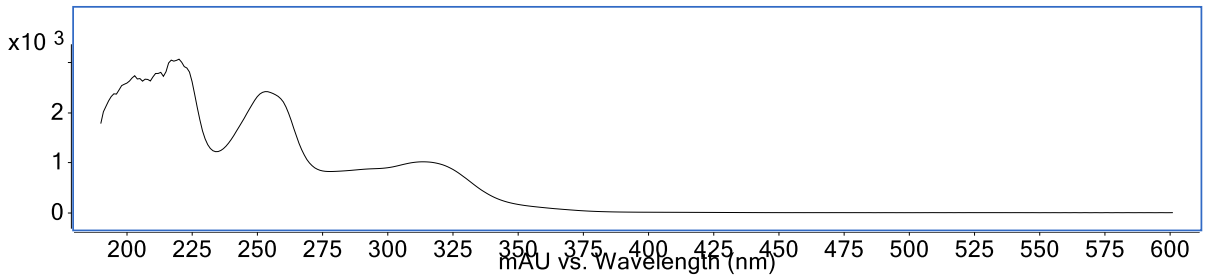

Figure 29: ***UV-vis spectrum of the purified pyochelin***

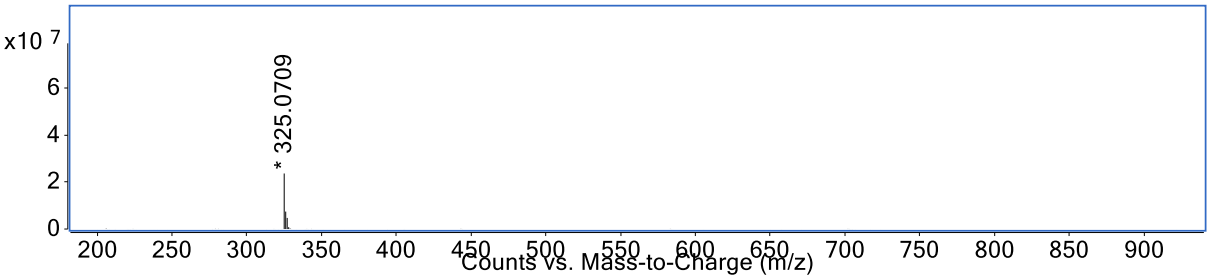

Figure 30: ***Mass spectrum of the purified pyochelin***

Finally, the purity of the molecules were roughly estimated with area ratio of the DAD spectrum (TWC) of the samples. The pyochelin reached an estimated purity of 78.7% whereas the pyoverdines purity reached 93.0%. Figure 32 illustrates the results of the purifity of pyochelin (A) and pyoverdine (B) .

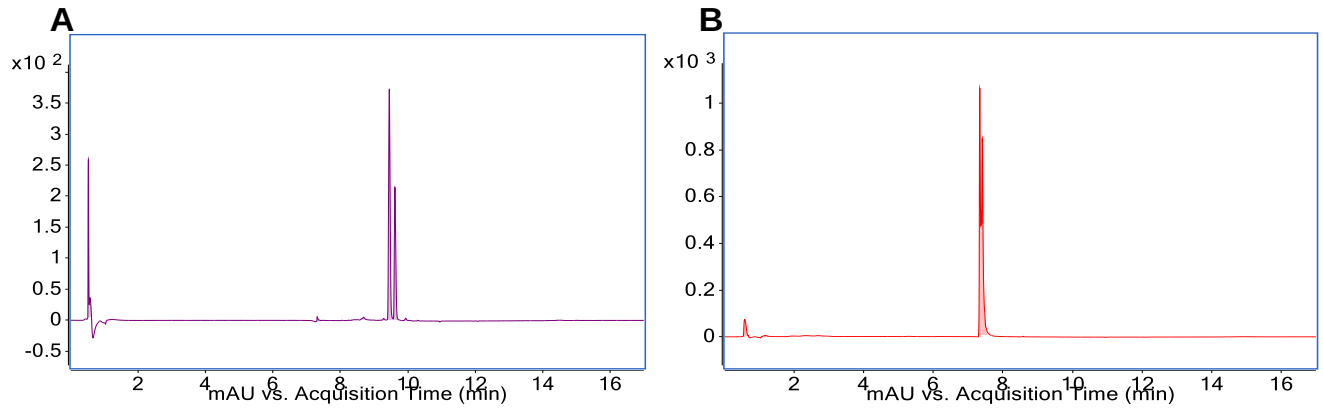

Figure 31: ***DAD spectrum (TWC) of*** *A:* ***purified pyochelin and*** *B:* ***purified pyoverdine***

5. Iron depletion induces the production of bacillibactin

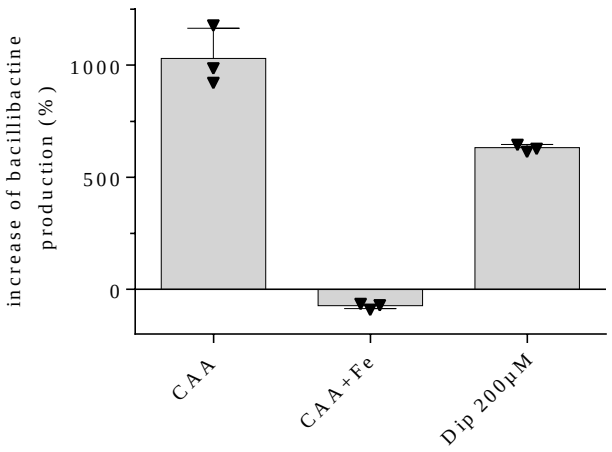

*Figure 32:**Iron deficiency induces the production of bacillibactin of*** **Bacillus velezensis GA1**. *CAA and CAA+Fe:* Bacillus velezensis *GA1 with 4% (v/v) of supernatant of* Pseudomonas *sp. CMR12a grown on CAA and iron supplemented CAA medium respectively. DIP 200µM:* Bacillus velezensis *GA1 culture with 200µM of DIP*

6. Inhibitory effect of the DIP

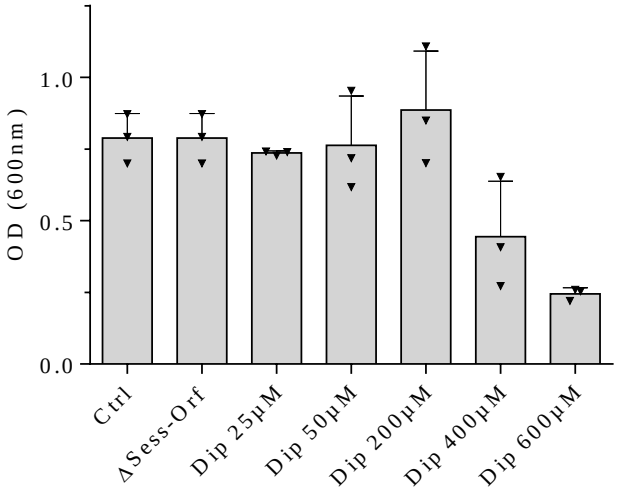

*Figure 33**: Growth Inhibition of iron chelatant**. The optical densitity of* Bacillus velezensis *GA1 culture is measured after 24h. ctrl: culture of* Bacillus velezensis *GA1 without DIP, Δsess-orf: culture* of Bacillus velezensis *GA1 with 4% (v/v) of* Pseudomonas *sp. CMR12a Δsess-orf supernatant.*

7. Siderophores of the Pseudomonas strains

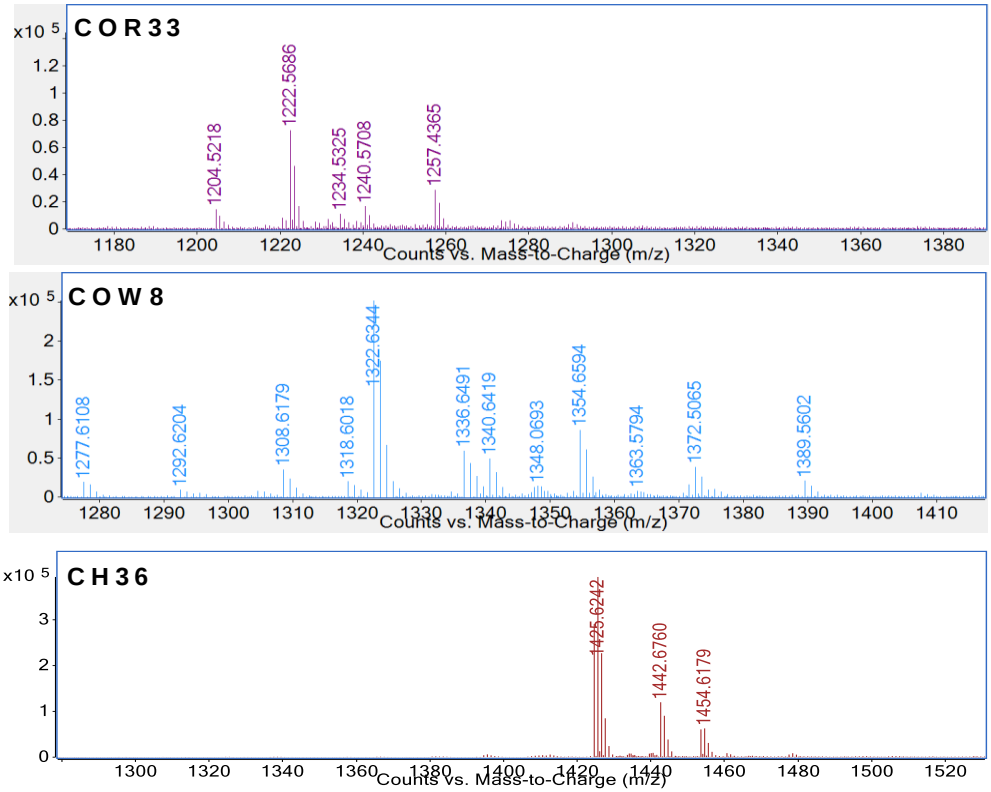

Figure 34: ***mass distributions of the pyoverdines of*** **Pseudomonas sp.** ***COR33, COW8 and CH36***

8. Iron quantification

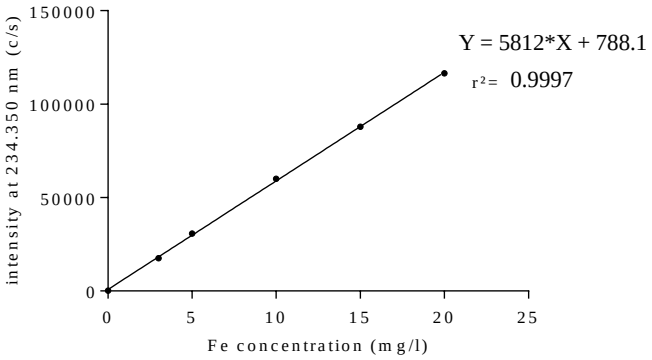

Figure 35: ***Iron calibration curve***

Table 10: ***Iron quantification of various media***

Sample	Iron content (mg/l)
Fresh CAA	<LOQ
Fresh Iron supplemented CAA	16.8
Spent CAA	<LOQ
Spent Iron supplemented CAA	0.663
Fresh Re ½	0.093