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THE LC-MS/MS PROFILING OF AHLs PRODUCED IN *SINORHIZOBIUM MELILOTI* NODULATING *ALYSICARPUS BUPLEURIFOLIUS*

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Abstract

We isolated *Sinorhizobium meliloti* (PCC21) from root nodules of wild legume species *Alysicarpus bupleurifolius* and characterized the AHL diversity produced in symbiosis. The TLC and HPLC-MS/MS profiles revealed production of a diverse array of AHLs that ranged from short chain C_4-HSL to long chain C_{12}-HSL. It was observed that isolates in young nodules produced AHL abundance. The study reports a novel symbiotic association between *S. meliloti* and *Alysicarpus bupleurifolius* and points to the possible presence of more than one synthase systems, as previously described in Rm1021 and AK63. The isolation of a strain producing wide ranging AHL molecules provides an opportunity to investigate the complex genetics of AHL production in *S. melitoti*.

Introduction

Bacteria use quorum sensing to coordinate its population locally. It may also occur among unrelated species regulating various processes at community level. Bacteria sense their quorum strength through signaling molecules called *auto inducer*s. Recently, attention has been paid to understand the nature of these signaling molecules. In most cases, the Gram-positive bacteria produce oligopeptides as signaling molecules while *N-Acyl Homoserine Lactones (AHL)* are produced in Gram-negative bacteria. A third family of signaling molecules known as *autoinducer-2* are produced both in Gram-negative and Gram-positive bacteria (Miller & Bassler, 2001).Shortly after, the protein like structures involved in quorum sensing was reported, when the *Lux* S orthologs were determined (Lewis *et al*., 2001). While in 2002, the receptor *Lux* P of *Vibrio harveyi* with its inducer AI-2 was also determined (Chen *et al*., 2002).

Hitherto a number of bacterial species are known to produce AHLs as signaling molecules. However, in many instances their genetics remains uncharacterized. One of such species is *Sinorhizobium meliloti*, a Gram-negative soil bacterium, which can establish a symbiotic relationship with multiple hosts: *Medicago sativa*; *Melilotus* and *Trigonella* (Gao *et al*., 2012). Besides establishing the symbiotic association between prokaryotes and eukaryotes through quorum sensing, a number of other processes such as virulence mechanism, antibiotic production, biofilm formation etc. involve extensive coordination of bacterial genes expressed globally (Mueller & Gonza´lez, 2011). Performing a wide range functions, these strain are expected to produce diverse AHLs (Marketon & Gonza´lez, 2002b; Teplitski *et al*., 2003).

Two AHL production systems *Sin* R and *Exp*R have been reported in *S. meliloti* (Marketon & Gonza´lez, 2002b).*Sin*R system controls the production of long chain AHLs. *S. meliloti* strain Rm8530 uses the AHL synthase *Sin*I to produce at least seven high molecular AHLs (Marketon *et al*., 2002). The production of a diverse array of AHLs suggests the presence of quorum sensing in *S. meliloti*, largely in the manner as in *R. etli* and *R. leguminosarum*. Earlier studies also showed that two strains Rm1021 and AK63 being well characterized, produce diverse AHLs (Marketon *et al*., 2002). The objective of the present study was to characterize the AHL diversity produced in *Sinorhizobium meliloti* found in symbiosis with the wild leguminous species *Alysicarpus bupleurifolius*.

Materials and Methods

Nodule isolation and growth condition: Roots of *Alysicarpus bupleurifolius* with nodules were collected from QAU campus in November, 2012. The healthy nodules were washed and detached from the roots under aseptic conditions. These were surface sterilized using 0.8% mercuric chloride for 2 minutes and washed twice with autoclaved deionised water. Nodules were macerated on sterilised glass slide containing a drop of water and the suspension was streaked on YEM agar plate with the help of sterilized inoculating loop. Plates were incubated at 28°C ± 2°C for 72 hours. Isolates were further streaked several times to get pure colonies. While the mutants were selected using streptomycin as the resistant source. For this purpose all strains were grown on TY-medium at 28°C ± 2°C.

Bacteriocin-type growth inhibition test: The indicator strain PRLIJ1, referred to as A34 (Wilkinson *et al.,* 2002)was suspended in 10ml TY broth to get an OD_{600}-0.4, mixed with 200 ml of TY agar and poured as 3 to 5 mm thick layer on a petri dish. The agar was allowed to settle and was overlaid with a thin layer of sterile TY agar. The growth inhibition was assessed for the isolates PCC1, PCC21 and the standard strains A31 and A34. The activity was measured as halos after 2 days of growth at 28°C (Table 1).

Table 1.The bacterial strains/isolates used in the study.

Strains	Description	Source
A34	*R. leguminosarum*derivative of 8401 carrying pRL1JI	Downie *et al*., (1983)
A31	*R. leguminosarum* strain lacking a symbiotic plasmid	Lamb *et al*., (1982)
Pcc1	*R. leguminosarum* strep^r Isolates from pea nodules.	This work
Pcc21	*Sinorhizobium meliloti* strep^r isolated from *Alysicarpus bupleurifolius*	This work
Cv026	*Chromobacterium violaceum*: a bio-indicator strain	McClean *et al*., 1997

AHLextraction and TLC: The isolate PCC21 was inoculated on Ty agar plate overlaid by the indicator strain *Chromobacterium violaceum* (CV026) and incubated at 28°C for 48 hrs. The samples were centrifuged at 15000g and AHLs were extracted from the supernatant using dichloromethane and dried with liquid nitrogen. AHLs were then dissolved in acetonitrile (Winson *et al*., 1995).The aluminium backedRP-18 (Merck) TLC plates were spotted with the AHL extracts. The samples were separated using methanol/water (60:40). The plate was removed from the tank, dried before elution. For colour development, TLC plates were overlaid by CV026 and incubated at 28°C for 48 hours (Shaw *et al*., 1997). The appearance of purple colour indicated the presence of AHLs in the media (McClean *et al*., 1997).

Mass spectrophotometry: The mass spectrometry was performed in a LTQ-XL mass spectrophotometer (Thermo Scientific). Foroptimization, an analysis was performed using the reference strain A34 and standard AHLs:*N*-acyl-homoserine lactone, *N*-dodecanoyl, DL-homoserine lactone (C12-HSL) and *N*-3oxo decanoyl-L-homoserine lactone (3oxoC10-HSL). Stock solutions of the analytes were prepared by dissolving the substances in methanol or acetonitrile at a concentration of 1 mg/ml and stored at-20°C. Standard solutions were prepared by diluting the stock solution to the desired concentration with acetonitrile acidified with 0.1% formic acid.

For mass spectrometric detection, the extracted AHLs were purified by solid phase extraction (SPE) as optimized by Li *et al*., (2006) using Strata C18E cartridge (Phenomenex). AHL extracts were dried in a vacuum drier and dissolved in 25/75 (v/v) Acetonitrile/water. The C18-RP cartridge was conditioned with 2ml each of HPLC grade water and methanol sequentially. The samples were loaded onto the column and washed with 15/85 (v/v) methanol/water and the analytes were eluted with 25/75 (v/v) hexane/isopropanol. Elutes were dried using liquid nitrogen, stored at -20°C and redissolved in 500µl LCMS grade acetonitrile prior to Mass spectrometric analysis.

MS system was tuned using two standards (3oxoC10-HSL and C12-HSL) before optimizing the analysis for AHLs. Tuning included ESI source parameters (probe position, gas flow, capillary temperature and tube lens voltage), voltage of ion optics and MS parameters. Probe position was set manually to the point where maximum intensity of ion signal was achieved. Sheath and auxiliary gas flow rates were adjusted to get stable spray. Voltage of ion optics was optimized to get maximum focusing of M+H ions of AHLs. MS parameters such as microscan, maximum injection time, ion isolation width and collision energy were optimized to get maximum signal strength of AHLs in the mass spectra. After tuning, the setting file was saved and used for AHLs analysis. The samples prepared by SPE were injected to the ionization source of the mass spectrometer with a syringe pump. Direct infusions of samples were carried out for the complete profiling of AHLs produced by each test strain. AHL ions signal were analysed in positive mode only.

The ESI-mass spectra obtained were used to characterize the AHL ionization behaviour. The $[M + H]^+$, $[M + H_2O]^+$ and $[M + Na]^+$ ions were monitored for AHLs. In addition, the ESI-MS/MS fragmentation behaviour of identified peaks of AHLs was investigated. By using MS/MS the structures of the AHLs produced were identified.

Results

Phenotypic observations: We observed an average of 5 to 6 nodules in *Alysicarpus* roots. Generally the nodules were small in size and pink in colour. When streaked on TY-medium, the colonies appeared round, shiny and gummy and the isolates were prolific and fast growing.

Identification of PCC21: To differentiate *Sinorhizobium* from *Rhizobium* small bacterioc in test was performed. The test is based on the growth sensitivity to 3OH-$C_{14:1}$-HSL, known as small bacterioc in (Gray *et al*., 1996; Schripsema *et al*., 1996). It was observed that no halozone formation took place in case of PCC21 which depicted that the isolate did not produce 3-OH-$C_{14:1}$-HSL, thuslacks pRL1JIplasmidwhichindicated its non-Rhizobial nature (Fig. 1).The 16S rRNA sequence amplified forPcc21 revealed 99% homology with *Sinorhizobium meliloti*. The sequences obtained were submitted to Gene bank (NCBI) under the accession number KC616316.Based on these results we report here a novel symbiotic association of *S. meliloti* with *Alysicarpusbu pleurifolius*.

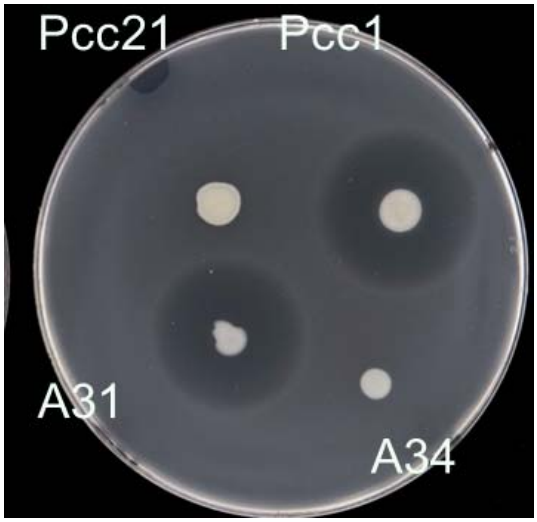

Fig. 1.The small bacteriocin test performed on a lawn of indicator strain A34with two days of incubation at 28°C. No holozones show growth sensitivity of PCC21 and A34, while halozone presence indicates activity in A31 and PCC1.

Characterization of AHLs produced by PCC21: The extract resolved on TLC plate overlaid by the bio-reporter strain *Chromobacterium violaceum* (CV026) showed that PCC21 produced diverse range of AHL molecules. The mobility pattern was revealed similar to the standards of known sizes (C6, C7 and C8Merck). The results further showed variation in the quantities which depicted greater production of C_6 and C_8AHL molecules while that of C7 was substantially low in quantity (Fig. 2). However, the TLC study remained limited in precise quantification of AHLs.

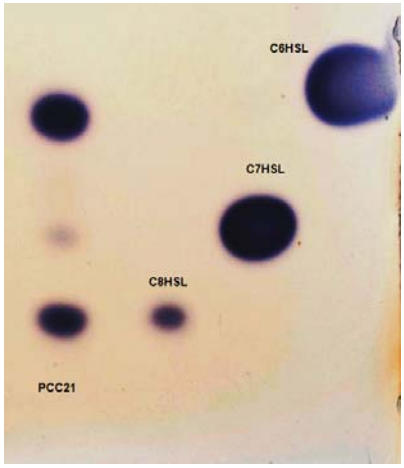

Fig. 2. Thin Layer Chromatography detecting moderate size AHLs in PCC21 extract with an overlay of *Chromobacteriumviolaceum* (CVO26) and standards of known size (Merck) as indicated.

The ESI MS/MS spectra of AHL analysis performed for PCC21 and the mass to charge (m/z) ratio (Table 2), showed diverse peaks and fragmentation pattern. The ratio of the peak height observed for the precursor ions in the neutral loss scan (neutral loss of mass 101) to that of the precursor ion observed in the parent scan (peak of 102) was characteristic of the functionality present in the fatty acid moiety. The m/z 172 $[M+H]^+$ and m/z 190 $[M+HOH]^+$ depicted the presence forC_4-HSL as the shortest chain of AHLs in the extract (Fig. 3a).The peak with m/z 202 $[M+H]^+$depicted the presence of 3OH-C_5-HSL (Fig. 3a). The peak of m/z 220reflected the presence of H_2O molecule adduct. Similarly the moiety with m/z 216 $[M+H]^+$ and m/z 201$[M+H]^+$ confirmed the presence of 3OH-C_6-HSL and C_6-HSL respectively. The peak with m/z 228$[M+H]^+$ showed the presence of C_8-HSL. The long chain molecules were identified as 3-oxo-C_{10}-HSL, C_{11}-HSL and 3-oxo-C_{12}-HSL (Fig. 3b).

In the present study, we observed a mechanism that was consistent with the loss of a water molecule and the subsequent formation of the corresponding3O-HSL. A chromatogram of 3O-C_{10}-HSL and 3O-C_{12}-HSLwas recorded and each target analyte was uniquely identified by the parent-to-product ion mass transition, i.e., 288.2 → 270.2 and 316.2 → 298.2 for 3O-C_{10}-HSL and 3O-C_{12}-HSL, respectively (Fig. 3b). This further supported the occurrence of hydrolyzed acyl containing homo serine-lactones in the extract.

Table 2. A comparison of theoretical and observed molecular mass of AHL moieties produced in *S. meliloti*.

Analytes	Chemical formula	Exact mass (Da)	$[M+H]^+$
C_4HSL	$C_8H_{13}NO_3$	171.09	172.1
3OH-C_5HSL	-	219	220.08
C_6HSL	$C_{10}H_{17}NO_3$	199.12	200.17
3OH-C_6HSL	$C_{10}H_{17}NO_4$	215	216.17
C_8HSL	$C_{12}H_{21}NO_3$	227.15	228.02
3oxo-C_{10}HSL	$C_{14}H_{23}NO_4$	269.16	270.1
3OH-C_{10}HSL	$C_{14}H_{25}NO_5$	287.17	288.17
C_{11}HSL	$C_{14}H_{23}NO_4$	269.16	270.1
3-oxo-C_{12}HSL	$C_{16}H_{27}NO_4$	297.19	298.42
3OH-C_{12}HSL	$C_{16}H_{29}NO_5$	315.20	316.08

Discussion

***S. meliloti* colonizing multiple hosts:** The diversity of plants species in Pakistan (Ali & Qaiser, 1986) and elsewhere especially in the semiarid zones offers huge opportunity to excavate and exhibit novel associations (Shinwari & Qaisar, 2011; Shinwari *et al.,* 2012) such as we have found in this study for *S. meliloti*. This depicts the significance of natural associations developed to mitigate harsh environment where these plant species have thrived over millennia and learned to communicate and benefit out of their associations with microbes especially bacteria. The present study is an example as to how microbes play their part in retiring natural stresses on host plants. The association of *S. meliloti* with *Alysicarpus bupleurifolius*, points to the fact that this species is symbiotically efficient and non-specific in case of host as *S.meliloti*is already known to have symbiotic association with *Medicago*, *Melilotus* and *Trigonella* (Gao *et al*., 2012). Hence it may develop its association with multiple hosts and it is expected that it might have association with a wide variety of leguminous species.

The diversity of AHLs produced in *S. meliloti*: It is well-established that bacteria nodulating legumes produce signaling molecules to coordinate through a process called quorum sensing (Fuqua *et al.*, 1994). Previous studies revealed these signaling molecules arediverse.

Consequently such microbes get a passport to interact with multiple hosts and are involved in regulating diverse processes (Winson *et al*., 1995; Khan *et al.,* 2012; Khan *et al.,* 2012a). Quorum Sensing in *S. meliloti* is complex and only partially characterized (Gao *et al*., 2005). The *S. meliloti* strain 1021 produces diverse N-acyl homoserine lactones (AHLs) however, there is no single reason for production of diverse AHL. It is likely that different culture conditions as described in Tepelistski *et al*., (2003) are responsible. Genetically speaking, *S. meliloti* has *Sin* Isynthase that produces long acyl side chains including C_{12}-HSL and bigger molecules. Marketon *et al.,* (2002a) reported that *S. meliloti* strain also uses a second and rather uncharacterized AHL-synthase to produce AHLs with shorter acyl side chains, including C_6-HSL etc. In the present case, we identified at least ten different AHLs including three short chain C_4-HSL, 3OH-C_5-HSL and 3OH-C_6-HSL, C_8-HSL and relatively small amount of C_6-HSL. We have also identified 3OH-C_5-HSL which was not reported previously in the above mentioned studies. Besides three long chain molecules were also observed as also reported previously by Marketoon *et al*., (2002a) and Teplitski *et al*., (2003) (Table 2). The production of wide variety of AHLs suggested that the strain has a diverse genetic background and possibly carries both *Sin* and *Exp* synthases that confer diverse capacities in this strain. However, this needs probing it genetically.

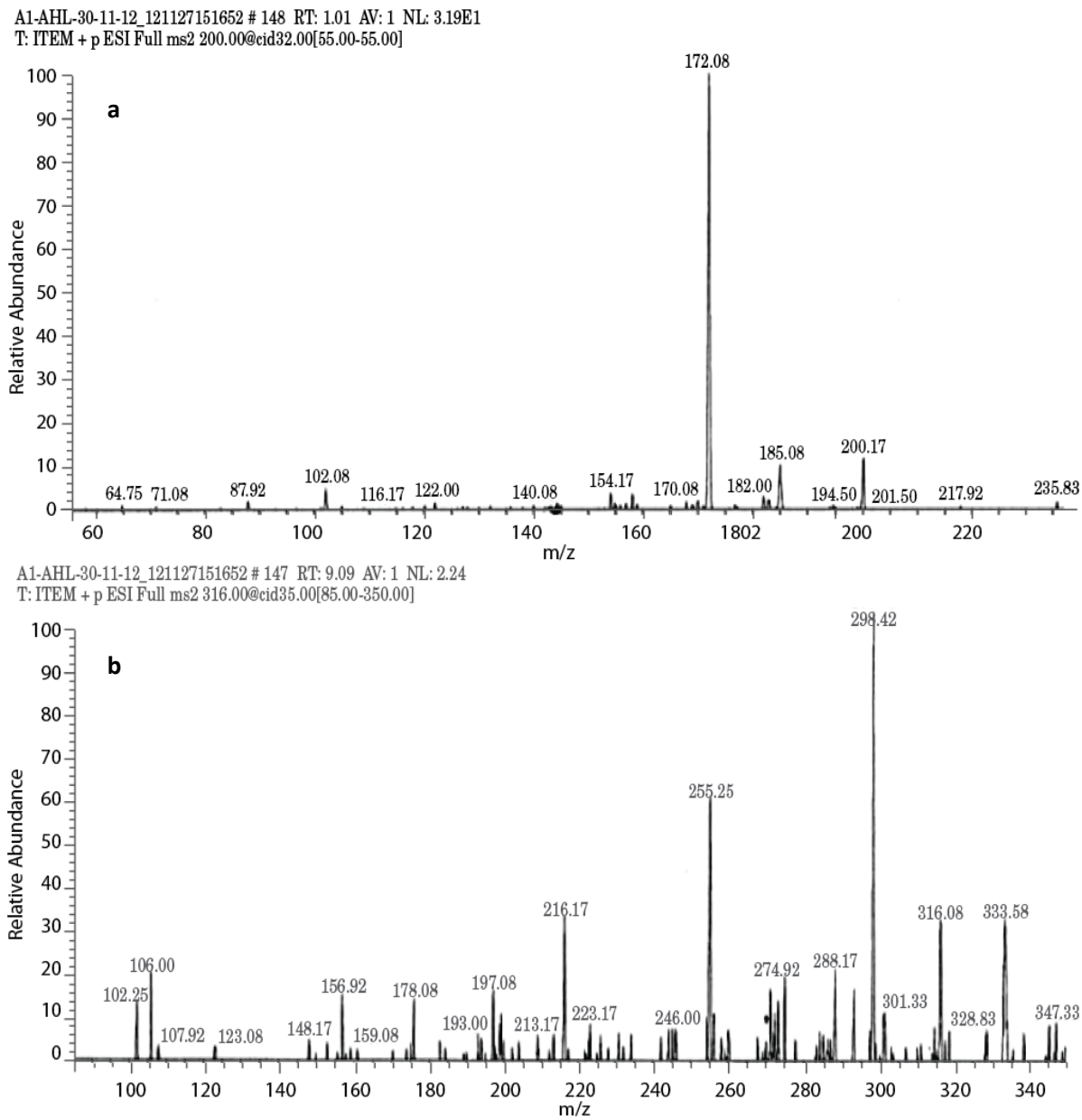

Fig. 3. Mass spectra of AHLs identified in PCC21 spent culture. (a) Short chain AHLs as depicted by the peaks: m/z 172 and 190 = C_4-HSL; m/z 202 = 3-OH-C_5-HSL; m/z 220 = C_5-HSL with a H_2O adduct; (b) m/z 216 = 3OH C_6-HSL; m/z 228 = C_8-HSL; m/z 270 = 3O C_{10}-HSL and m/z 298 = 3O C_{12}-HSL

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