Sugarcane and Grapevine Endophytic Bacteria: Isolation, Detection of Quorum Sensing Signals and Identification by 16S v3 rDNA Sequence Analysis

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One quorum sensing (QS) system in bacteria measures diffusible signal molecules called *N*-acyl-homoserinelactones (AHLs). At threshold concentrations of AHLs, activation or repression of genes as a function of the cell population density can coordinate disease progression or symbiosis in eukaryotic bacterial pathogens. The production of AHLs by plant bacterial endophytes has not been investigated even though they are commonly isolated from internal plant tissues. In this paper, we isolated and identified endophytes from sugarcane and grape plants, to determine if the endophytes produce AHL signals. Samples were collected from stem tissue of sugarcane and from xylem fluids of grapevine. Culturable bacterial isolates were purified by repeated subculture and the genus and / or species of the endophytes were identified by sequence analysis of the v3 (variable region) of the 16S rDNA gene. Using these analyses six unique isolates from sugar cane and seventeen isolates from grape xylem sap were identified. The production of QS signals by the sugarcane and grape endophytes were tested using five different AHL-dependent bacterial biosensors strains. Among the endophytic strains identified, five of six sugarcane and fourteen of fifteen of grape elicited a significant response in at least one AHL-dependent biosensor.

Keywords: quorum sensing, endophytes, acyl-homoserine lactones, rDNA

1. Introduction to bacterial endophytes.

Plant-microbe interactions may be neutral, pathogenic or beneficial to the host. Bacterial endophytes are bacteria that colonize the internal tissue of the plant while showing no sign of infection or negative effect on the host. Endophytic bacteria are ubiquitous in most plant species either actively colonizing or latently dormant. The diversity of culturable bacterial endophytes is exhibited not only in the variety of plant species colonized but also in the many taxa involved with some being members of the common soil bacteria genera including *Azospirillum, Bacillus, Burkholderia, Enterobacter,* and *Pseudomonas.* Endophytes may either become localized at the point of ingress or spread throughout the plant. Endophytes have been shown to reside within cells, in the intercellular spaces or in the vascular systems of phloem and xylem [1, 2].

Bacterial endophytes colonize an ecological niche similar to phytopathogens and thus have been used as biocontrol agents in the control of bacterial and fungal plant pathogens as well as insect and nematode plant pests. Bacterial endophytes can act to promote seedling emergence, overall growth, and plant growth under stress conditions. These attributes have been credited to the ability of endophytes to produce novel compounds and antifungal metabolites in order to survive in the host environment. Consequently, the opportunity to find new endophytic microorganisms among the diversity of plants in different ecosystems is intriguing and may lead to the identification of novel compounds for drug development in the treatment of human diseases, for industrial applications or to enhance agriculture production [1-3].

2. Bacterial *N*-acyl-homoserine lactone communication signals.

It is not known if bacterial communities inside plants communicate. It has been speculated that the beneficial effects of bacterial endophytes in the host are the result of the combined effects of their activities [3]. In this regard, the production of communication signals by bacteria endophytes in the process of quorum sensing (QS) has not been reported. In addition, it is not known if endophytes can communicate using QS and /or whether some endophytes can detect the signal (eavesdrop or listen in) but not contribute to its production (communication). The QS mechanism enables bacteria to coordinate responses at the population level [4, 5]. As the bacterial population cell density increases, the accumulation of QS signals trigger physiological responses in the bacterial population (know as a quorum). Such responses foster energy conservation, the production of antibiotics, virulence factors, exo-enzymes and biofilm development. Other QS regulated responses include factors that enhance symbiotic interactions between a bacterium and its host plant species [6-9]. The QS signal sensed in these examples are the *N*-acyl-homoserine lactones (AHLs)[10]. These molecules are freely diffusible, and all share the core homoserine lactone head group but contain distinct acyl side chains. The acyl side chain varies by the number of carbons, substitutions on the third carbon and the presence of a double bond (Fig. 1) [11, 12].

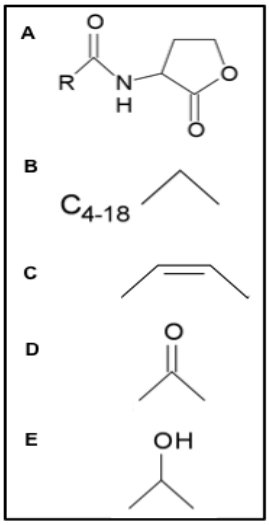

Fig. 1 Representative chemical structures of *N*-acyl-homoserine lactone quorum sensing signal molecules. Core molecule, A; variation in acyl side chain length, B; presence of double bond in side chain, C; oxo substitution at 3rd carbon of side chain, D; and hydroxyl substitution at 3rd carbon of side chain, E.

Our research goal was to determine if grapevine xylem fluid (GXF) and sugarcane stem tissue (SST) harbor culturable bacterial endophytes and begin to investigate AHL QS signal synthesis in the isolates. In this communication, we identified culturable bacterial endophytes isolated from GXF and SST by DNA sequence analysis of the v3 (variable region) of the 16S rDNA gene (Table 1). This was achieved by using five complementary AHL-dependent bacterial biosensors to test for AHL signal production [13].

3. Plant tissue collection and the isolation of grapevine xylem fluids (GXF) and sugarcane stem tissue (SST) endophytes.

Grapevine xylem fluid was collected from a 5-year old Riesling (*Vitis vinifera*) grafted on rootstock Courderc 3309 at an orchard at the Cornell Agricultural Experiment Station, Geneva, NY. Approximately 10 mL of GXF was collected from 21 grape plants. Sugarcane stem tissue was obtained from the West Indian Sugarcane Company (WISCO) located in Westmoreland Jamaica. Sugarcane (*Saccharumsps*.) stem tissue of approximately 5 inches was surface sterilized with 20% sodium hypochlorite for 10 minutes followed by two 10-minute washes of sterile distilled water.

One hundred µL of each GXF sample was plated onto potato dextrose bacterial agar medium (PDA). From the GXF bacteria recovery medium (PDA), 30 distinct bacterial colonies were identified, isolated and re-plated to produce pure colonies. To isolate SST endophytes, internal tissue was dissect under sterile conditions and place into 100 mL of PD broth media and incubated at 30^{o}C for 48 hours with shaking at 250 rpm. To facilitate isolation of pure colonies from SST, serial dilutions were conducted from 10^{-1} to10^{-10}. One hundred µL of the samples ranging from 10^{-5}-10^{-10} was plated onto five different agar media: Luira broth, nutrient agar, potato dextrose, colony isolation, and R2A (Fig. 2).

3.1 Collection of GXF and SST endophytes.

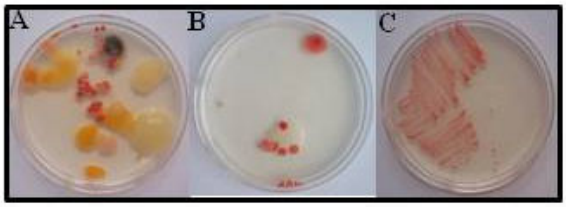

Fig. 2 Sample of GXF endophytes recovered on PDA medium and strain purification. GXF isolates recovered from grape plants in row 1, plant 19 (R1/19) and R3/13, respectively, A and B; purified endophyte isolate GXF4 isolated from grape plant R1/19, C.

3.2 Genomic DNA isolation.

Pure bacterial isolates were harvested from overnight grown cultures in microcentrifuge tubes and resuspended in 567 µL TE buffer, 30 µL of 10% SDS and 3 µL of 20 mg/mL proteinase K which were mixed and incubated at 37^{o}C for 1 hour. Following incubation, 10 µL of 5 M NaCl was added and mixed, then 80 µL CTAB/NaCl solution was added and the mixture was incubated at 65 ^{o}C for 10 minutes. An equal volume of chloroform/isoamyl alcohol was added and centrifuged for 5 minutes at maximum speed in a microcentrifuge. The aqueous phase was transferred to a new tube and the genomic DNA was extracted with phenoly/chloroform/isoamyl alcohol. The genomic DNA was precipitated with 0.6 volumes of 100% isopropanol, washed three times with 70% ethanol ($^{v}/_{v}$) and resuspended in 50 µL of TE buffer.

3.3 PCR amplification of the 16S v3 rDNA region.

The v3 region was amplified using: twelve picomoles of forward and reverse primer, 1 mM $MgSO_4$, 0.5 mM of each of the four deoxynucleotide triphosphates, 0.2 ng of genomic DNA and 1 unit of Platinum *Pfx* DNA polymerase (Invitrogen) using the following PCR conditions: 1 cycle at 94°C for 2 minutes, followed by 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 1 minute. The forward and reverse primers used to amplify the v3 region were 5'-ACTCCTACGGGAGGCAGCAG-3' and 5'-ATTACCGCGGCTGCTGG-3'.PCR amplicons were resolved by electrophoresis on 0.8% ($^{w}/_{v}$)agarose gels followed by gel extraction using the QIAquick Gel Extraction Kit (Qiagen) to prepare the samples for nucleotide sequencing.

Table 1. SST and GXF endophytes, their identification by partial 16srRNA gene sequencing, accession number of highest homolog in NCIB database and the production of acyl-homoserine lactones.

Bacterial Endophyte Isolate	Identification / DNA Homology	Homology Accession Number	AHL Production (number positive of five biosensors)
SST1, 5	*Bacillus thuringiensis* isolate S11	FJ655837.1	2
SST2, 12, 13	*Erwiniasp*. GS-1-08	FJ816023.1	3
SST3, 4, 7, 10	Unclassified Gammaproteobacterium of Sugar cane weevil, *Candidatus nardonell*	FJ626345.1	3
SST6	*Enterobacter aerogenes* strain 1-WCH; Bacterium in the red turpentine beetle, *Dendroctonus valens* LeCont	FJ811873.1	1
SST8	No similarity found	-	1
SST11	*Bacillussp*. isolate JZDN22	DQ659002	0
GXF1, 2, 9, 10	*Curtobacteriumsp*. B18 gene	AB027699.1	4
GXF3	*Salmonella entericasubsp. arizonae* strain GTC1732	AB273736.1	1
GXF4, 19, 26	Environmental sample, clone N2 S4E16f	AB485318.1	4
GXF6	Environmental sample, Clone nbw312f08c1	GQ088421.1	1
GXF8, 14, 25	*Flavobacteriumoceanosedimentum* strain ATCC 31317	EF592577.1	2
GXF11	*Arthrobacter sp*. DJWH1	EF694316.1	2
GXF12	*Staphyloccocussp*. HB2	AM268421.1	0
GXF13	*Brachybacteriumsp*. YACS-41	DQ649451.1	3
GXF15	*Bacillussp*. YACS13	DQ658919.1	4
GXF17	*Bacillussp*. YANN14	DQ658905.1	4
GXF18	Uncultured *Bacillussp*. clone QJHW03	FJ384483.1	4
GXF21	Environmental sample, clone FD01B10	FM873290.1	1
GXF22	Environmental sample, clone nbw528d04c1	GQ105510.1	2
GXF27	*Actinomycessp*. YACS-36	DQ649438.1	2
GXF28	*Streptomyces sp*. 81	FJ754313.1	1

3.4 Identification of bacteria.

The nucleotides corresponding to the v3 16S rDNA region was sequenced in both directions using the primers 5'-ACTCCTACGGGAGGCAGCAG-3' and 5'-ATTACCGCGGCTGCTGG-3. The sequenced data from the amplified v3 region were compared to the sequences deposited in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) using the blastn algorithm to determine the best sequence match [14].

It should be noted that six of the SST and GXF isolates failed to share sequence homology in the NCBI database. Isolate SST3, 4, 7, 10 gave identity to an unclassified endosymbiont of the sugarcane weevil, SST8 shared no homology with 16S sequences deposited in the database, and isolates GXF4, 6, 21 and 22 shared homology to environmental samples from a metagenomic study (Tables 1).

4.Quorum sensing signal production by endophytes: culture of endophytes.

Overnight cultures (5 mL) were prepared from each of the GXF and SST endophytes purified from the recovery medium. An equal volume of acidified ethyl acetate (aEtOAc) was added to the overnight cultures and the mixture was agitated for 30 minutes at room temperature at 150 rpm. The tubes were then centrifuged to separate the aqueous from the aEtOAc phase. Under these conditions, AHLs partition into the non-polar aEtOAC phase. The aEtOAc was aspirated off and then transferred into 1.5 mL microcentrifuge tubes, dried in a Savant speed-vac and resuspended in 30 µL of aEtOAc. This produced 20-fold concentrated aEtOAc extracts. These extracts were used in AHL detection assays.

4.1 AHL-dependent bioassays for screening for quorum sensing signals.

"T"-Streak or pigment bioassay. In "T"-streak assays, the *Chromobacterium violaceum* colorless mutant, CV026 was used [15]. In the presence of exogenous QS signals CVO26 produces the purple pigment (violacein), indicating the presence of AHL in the sample. *C. violaceum* wild type strain was used as a positive control. *E. coli* DH5α was the negative control in the T-streak plate assays. The biosensor, controls, and samples were grown on tryptone – yeast extract medium mixed with PDA medium using a ratio of 1:1 (v/v). Each isolate was tested at least two times using the "T"-streak bioassay.

*Bioluminescent or light biosensor bioassays.*The AHLs from the bacterial samples were detected using bioluminescence reporter plasmids pSB401, pSB536, pSB1075 in *E. coli* stain JM109, and in the*Agrobacterium tumefaciens* strain A136 containing (pCF218) and (pMV26) [16-19]. A list of the bacterial biosensors, their AHL receptor and cognate QS signal is given in Table 2.

An overnight culture of these five biosensors were grown in LB with appropriate antibiotic and diluted 1:10 in LB and 200 µl of the diluted cell suspension was added to the round bottom tubes (12 x 50 mm) containing dried aEtOAc samples. Tubes were incubated at 30°C with shaking for 5 to 6 hours before bioluminescence was measured using a Turner Designs TD 20/20 luminometer. The TD 20/20 luminometer was adjusted to different sensitivities due to the varying response of the JM109 series of biosensors to their cognate AHL signal. Relative light unit measurements were made at 30.0, 39.9, 50.1 and 30.0% sensitivity for LuxR-, AhyR-, LasR- and TraR-based biosensors, respectively as previously described by our laboratory [16-18].

*Thin layer chromatography (TLC) with biosensor detection.*Reverse-phase (RP) TLC plates were used to determine AHL signal profiles. Concentrated aEtOAc extracts were spotted at the origin in 2 µl volumes and from one-half to two ml supernatant equivalents were loaded per lane onto a C18 RP-TLC plate (EMD Chemicals, Inc. Gibbstown, NJ). Plates were developed in a 70% methanol:water mobile phase, dried and AHLs were detected using biosensor NTL4 (pZLR4) overlay as described[16-18]. AHL signals were identified with appropriate reference compounds. This involves determining and comparing retardation factors (Rf) of unknown samples to AHL reference compounds [13, 15]. TLC analyses were repeated at least twice.

Table 2. Bacterial biosensor strains used in this work.

Biosensor strain	Receptor	Cognate AHL[1]	Bioassay[2]	Ref.
A. tumefaciens A136 (pCF218, pMV26)	TraR	3-oxo-C8-HSL	Light	13
C. violaceum CV026	CviR	C6-HSL	Pigment	15
E. coli JM109 (pSB401)	LuxR	3-oxo-C6-HSL	Light	19
E. coli JM109 (pSB536	AhyR	C4-HSL	Light	13
E. coli JM109 (pSB1075)	LasR	3-oxo-C12-HSL	Light	19
A. tumefaciens NTL4 (pZLR4)	TraR	3-oxo-C8-HSL	β-galactosidase	13

[1]Cognate AHL as follows:C4-HSL, *N*-butanoyl-homoserine lactone; C6-HSL, *N*-hexanoyl-homoserine lactone; 3-oxo-C6-HSL, *N*-3-oxo-hexanoyl-homoserine lactone; 3-oxo-C8-HSL, *N*-3-oxo-octanoyl-homoserine lactone; 3-oxo-C12-HSL, *N*-oxo-dodecanoyl-homoserine lactone.[2]Describes the AHL-dependent bioassay type [13].

4.2 Biosensor responses to endophyte extracts.

Four AHL-dependent bioluminescence-producing biosensors containing different AHL receptors (Table 2) were used to screen ethyl acetate extracts of the SST and GXF endophytes for AHL molecules. The majority of our isolates (19 of 21) induced significant light production (Table 3) by at least one of the light-producing biosensors. Different biosensors will detect different AHL molecules at different sensitivities, thus overlap exist in detection of AHLs using different biosensors (Table 3) [13].

Table 3. Number of SST and GXF isolates activating a detectable phenotype in the biosensors.

Isolates	JM109 (pSB1075)[1]	JM109 (pSB401)[1]	JM109 (pSB536)[1]	A136[1]	CV026[2]
SST (of 6)	2	2	1	4	2
GXF (of 15)	9	8	4	11	1

4.3 AHLs produced by GXF4 endophyte.

To determine the number and size of the AHLs produced, thin layer chromatography (TLC) was used combined with signal detection using biosensor NTL4 (pZLR4). An example is shown using the isolate GXF4. Using this approach, GXF4 was found to produced three different AHLs, two showing Rf values identical to AHL standards C6-HSL and C8-HSL, and a third signal which migrates faster than C6-HSL (Fig. 3D). Further work is required to identify the AHL molecules produced by GXF4.

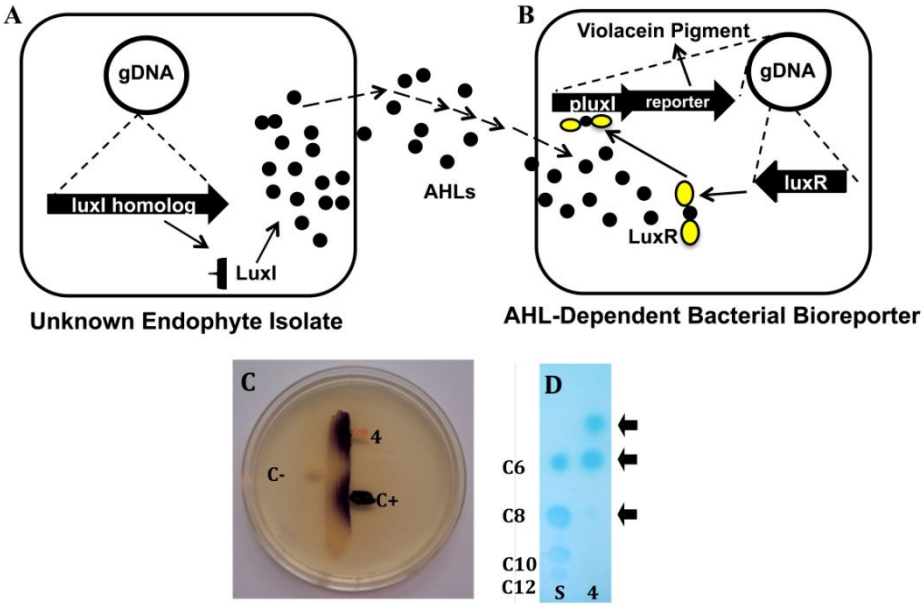

Fig. 3 Detection of AHLs from GXF4 endophyte. Bioreporter detection of AHLs from endophyte isolates. Diagram illustration of the production and diffusion of AHLs by unknown endophyte isolate, A; and the detection and response of bacterial biosensor CV026 to AHLs produced by adjacent endophyte, B. Abbreviations include: gDNA, genomic DNA; AHLs, *N*-acyl-homoserine lactones; *luxI*, AHL synthase homolog; *luxR*, AHL signal receptor homolog; *pluxI*, AHL responsive promoter; and reporter gene(s), violacein pigment production. When the AHLs produced by the bacterial streak of the unknown endophyte reaches a high concentration, the AHL binds to the LuxR homolog receptor protein (shown in yellow) which is a transcriptional activator. This causes the receptor protein to dimerize and adopt the active conformation that leads to the transcription of the reporter gene(s).Violacein pigment bioassay for AHL signal production by GXF4 (4) using T-streak bioassay and CV026 biosensor, C. Purple halo indicates AHL production. C-, negative control and C+, positive control strains. TLC separation and detection of AHLs from GXF4 used biosensor NTL4 (pZLR4) which activated the *lacZ* producingβ-galactosidase and detectable in the presence of X-gal as a blue spot, D. Lane S, reference AHLs, C6-HSL, C6; C8-HSL, C8; C10-HSL, C10; and C12-HSL, C12. Lane 4, extract sample from GXF4. Arrows indicate AHLs identified in GXF4 extract.

5. Conclusions

In summary, bacterial endophytes were isolated from sugarcane and grapevine plants. The production of communication signals of the AHL class, as determined by five AHL-dependent biosensors, appears to be common to these isolates. Further work to identify the signal molecules that induce the AHL-biosensors used in this study is warranted, especially with isolates identified that have not been historically shown to produce AHLs, e.g. Gram + isolates. Bacterial endophytes, in general, are a poorly examined group that are plentiful and historically a reliable source of novel bioactive compounds with potential for application in a variety of medical, industrial and agricultural

fields [3]. The determination that QS AHL molecules may regulate the synthesis of bioactive compounds by bacterial endophytes would trigger further attention in the study of the biology of bacterial endophytes.

The methods described in this manuscript can be used to design experiments with the aim of identifying endophytic or epiphytic bacteria. The procedures relating to the isolation and culturing of bacteria, gDNA isolation, PCR amplification, sequencing and bioinformatical identification of bacteria are of a general nature that would allow teachers and or researchers to develop experiments to identify bacterial organisms using 16S v3 rDNA sequencing. The senior authors of this manuscript (Hudson and Savka) have designed such activity in a newly designed course at the Rochester Institute of Technology in the School of Biological and Medical Sciences called Bio-Separations: Principles and Practices. The premise of the laboratory exercise is based on a form of active learning known as inquiry-based learning [20]. The core premise of this philosophy is that learning should be based on questions driven by students. For this laboratory exercise, students are encouraged to design the experiments by isolating and identifing bacteria from a plant(s) of their choice. The students do the entire process from the culturing to the identification using sequence analysis over a three-week period.

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