Research in Microbiology 163 (2012) 119–124

www.elsevier.com/locate/resmic

Response of leaf-associated bacterial communities to primary acyl-homoserine lactone in the tobacco phyllosphere

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Received 8 July 2011; accepted 3 November 2011
Available online 22 November 2011

Abstract

The phyllosphere is inhabited by large populations of epiphytic bacteria that are able to modulate their phenotypes and behavior by quorum sensing (QS). However, the impact of acyl-homoserine lactones (AHLs) involved in QS on the ecology of bacteria in their natural habitat remains unclear. Therefore, we used a bioassay and liquid chromatography–mass spectrometry to detect AHLs in the tobacco phyllosphere. Our results identified several AHLs in the tobacco phyllosphere, the majority of which were short-chain AHLs. Furthermore, the addition of an exogenous *N*-(3-oxohexanoyl) homoserine lactone (3OC6HSL), which is seen in the naturally occurring tobacco phyllosphere, generated variability in the composition of the bacterial community as determined by denaturing gradient gel electrophoresis (DGGE) analysis and phospholipid fatty acid (PLFA) analysis. Notably, the ratio of Gram-positive (GP) bacteria increased in response to treatment with 1 μM AHL, but decreased incipiently when treated with 10 μM AHL. These observations provide insight into the composition of the leaf-colonizing epiphyte community responsible for AHLs, particularly GP bacteria as they do not use AHLs as signaling molecules for QS.

Keywords: Phyllosphere; Acyl-homoserine lactone; Bacterial community; Epiphytes; Interaction

1. Introduction

Quorum sensing (QS) is a form of microbial chemical communication that relies on a cell density-dependent mechanism to modulate gene expression and alter bacterial behavior (Ng and Bassler, 2009; Waters and Bassler, 2005). By using a range of small, diffusible signal molecules, QS bacteria regulate a multitude of functions, including the production of virulence factors, bacterial motility, and symbiosis (Pacheco and Sperandio, 2009). *N*-acyl-homoserine lactones (AHLs) are a highly conserved class of QS signaling molecules used by a wide range of Gram-negative (GN) proteobacteria that inhabit diverse environments (Manefield and Whiteley, 2007). Importantly, plants that have been genetically modified to synthesize AHLs have the potential to affect bacterial processes regulated by QS signals (Fray et al., 1999). While much has been learned about QS using pure bacterial cultures, little is known about environmental AHLs and their ecological roles.

The phyllosphere, with a terrestrial leaf surface area estimated to exceed 10^8 km^2 globally, is considered an important habitat for microorganisms. Bacteria are the most common colonizers of this habitat and exist in populations of approximately 10^6–10^7 cells/cm^2 per leaf (Dulla et al., 2005). The overall microbiota in the phyllosphere is incredibly large and it should not be ignored. Phyllosphere microbial populations can also positively or negatively influence plant health and growth. Previous studies have focused mainly on bacterial plant pathogens that exhibit QS-dependent behavior, such as *Pseudomonas syringae* and *Erwinia carotovora* (Loh et al., 2002). Less is known about non-pathogenic microorganisms that inhabit the phyllosphere (Delmotte et al., 2009),

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doi:10.1016/j.resmic.2011.11.001

particularly their AHL-producing counterparts. Recently, Dulla and Lindow (2009) showed that at least 7% of the culturable bacterial epiphytes from leaves can synthesize AHLs, and suggested that widespread AHL-mediated cross-talk exists in the phyllosphere. However, current knowledge of naturally occurring AHLs in the phyllosphere and their impact on leaf-associated bacterial communities is scare.

The objective of our study was to assess both the presence of AHLs and AHL-mediated influence on the composition of the leaf-associated bacterial community in the tobacco phyllosphere. The composition of the bacterial community was analyzed using cultivation-independent analysis based on phospholipid fatty acid (PLFA) and denaturing gradient gel electrophoresis (DGGE) profiles. To gain insight into the changes that were identified, sequences that corresponded to the DGGE bands were also analyzed.

2. Materials and methods

2.1. Plant material and profiling of AHLs in the phyllosphere

The tobacco plant (*Nicotiana tabacum* L. cv. NC89) was used in this study. After 9 weeks (before the flowering stage) of plant growth in a greenhouse, leaf samples were randomly collected. Using an axenic technique, 30 g of mixed leaves were placed into sterile flasks with acidified EtAc (ethyl acetate containing 0.2% glacial acetic acid). After sonication for 10 min, the mixture was vortexed for 1 h. After centrifugation, the extracted supernatant was dried under N_2 gas and stored at −20 °C until processing for subsequent analysis. All sample extractions were performed in triplicate. The control samples were treated with the same procedure, except that the leaves were sonicated and rinsed using sterile purified water before extraction with EtAc.

Extracts with AHL-like activity were detected by cross-streaking against AHL bioassay strains *Agrobacterium tumefaciens* A136 (McLean et al., 1997) and *Chromobacterium violaceum* CV026 (McClean et al., 1997). AHL profiling was confirmed using a Waters Micromass Q-Tof micromass spectrometer (LC/MS) system. All samples were applied to a C18 column (ZORBAX Eclipse XDB-C18, 5 μm 250 × 4.6 mm; Agilent, USA) and eluted first using an isocratic profile of acetonitrile–water (60:40) for 15 min followed by a linear gradient from 60 to 100% acetonitrile in water over 5 min. The effluent was ionized by electrospray ionization (ESI) and detected in the positive ion mode (Morin et al., 2003).

2.2. Sample treatment and PLFA analysis

The experimental design was a randomized complete block with three replications for each of the AHL treatments tested. 9-week-old tobacco plants were sprayed with 1 μM or 10 μM *N*-(3-oxohexanoyl) homoserine lactone (3OC6HSL) and control treatments were sprayed with autoclaved distilled water. 3OC6HSL was selected because it was the primary AHL extracted from the tobacco plant phyllosphere (Table 1). Randomly collected leaves from the plants were sampled axenically every day for a week. Bacteria from leaves were collected for DNA extraction or PLFA analysis as described previously (Zhang et al., 2009).

The analysis of PLFA was performed as described previously with minor alterations (Bligh and Dyer, 1969). The composition of PLFA was determined using an Agilent 7890 GC (USA) on an HP-5 MS capillary column (J&W Scientific, USA) coupled with 5897 MSD. The relative quantities of individual fatty acid methyl esters were quantified using $C_{19:0}$ as a standard and converted to cell numbers as described elsewhere (Mohanty et al., 2006). Phospholipid fatty acids were assigned to taxonomic groups as categorized elsewhere (Myrold et al., 2005). Briefly, terminally branched saturated PLFAs (i14:0, a15:0, i15:0, i16:0, a17:0, i17:0) were indicative of GP bacteria, whereas some monounsaturated PLFAs (16:1ω7, 17:1, 18:1ω7) were indicators of GN bacteria. Analysis of variance (one-way ANOVA) for independent samples was performed using SigmaPlot 11.0 (Systat Software Inc., USA). The level of significance was determined using the Duncan test at $P < 0.05$ followed by original ANOVA results.

2.3. Denaturing gradient gel electrophoresis analysis

Total phyllosphere community DNA was extracted using a Fast DNA kit (Qbiogene, USA) according to the manufacturer's instructions. Purified total genomic DNA was used as the template for amplification of the V3 region of the small subunit rRNA gene using BA338fGC and UN518r primers (Lambais et al., 2006). PCR amplification was carried out as described previously (Zhang et al., 2009). DGGE analysis was performed with the Dcode Universal Mutation Detection system (BioRad, USA) according to the instruction manual. Purified PCR products were subjected to DGGE analysis using 8% polyacrylamide [acrylamide–bisacrylamide (37.5:1)] gels with a 40–55% denaturing gradient (100% denaturant containing 7 M urea and 40% formamide) and were electrophoresed at 60 V for 17 h at 60 °C. After imaging, DNA band positions and intensities were detected using Quantity One software (BioRad, USA). The resulting DGGE profiles were analyzed, and cluster analyses were performed using the Minitab software program (Minitab Inc., USA).

Representative bands from the DGGE gels were excised and transferred to tubes with 50 μl of sterile water for

Table 1
Acyl-homoserine lactones detected using LC–MS–MS in sample extracts from the tobacco phyllosphere.

AHL	Parent ion	Fragmentation ions		Concentration (pmol g^{-1})
	$[M + H]^+$ (*m*/*z*)	Lactone moiety (*m*/*z*)	Acyl-chain moiety (*m*/*z*)	
C6–	200	102	99	5.2
3-O–C6–	214	102	113	9.8
C8–	228	102	127	6.6
3-O–C8–	242	102	141	3.2
C14–	312	102	211	0.4

m/*z* = mass/charge ratio.