J. Microbiol. Biotechnol. (2007), **17**(2), 226–233

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY
© The Korean Society for Microbiology and Biotechnology

Production of Acyl-Homoserine Lactone Quorum-Sensing Signals is Wide-Spread in Gram-Negative *Methylobacterium*

POONGUZHALI, SELVARAJ, MUNUSAMY MADHAIYAN, AND TONGMIN SA*

Department of Agricultural Chemistry, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea

Received: August 2, 2006
Accepted: October 2, 2006

Abstract Members of *Methylobacterium*, referred as pink-pigmented facultative methylotrophic bacteria, are frequently associated with terrestrial and aquatic plants, tending to form aggregates on the phyllosphere. We report here that the production of autoinducer molecules involved in the cell-to-cell signaling process, which is known as quorum sensing, is common among *Methylobacterium* species. Several strains of *Methylobacterium* were tested for their ability to produce *N*-acyl-homoserine lactone (AHL) signal molecules using different indicators. Most strains of *Methylobacterium* tested could elicit a positive response in *Agrobacterium tumefaciens* harboring *lac*Z fused to a gene that is regulated by autoinduction. The synthesis of these compounds was cell-density dependent, and the maximal activity was reached during the late exponential to stationary phases. The bacterial extracts were separated by thin-layer chromatography and bioassayed with *A. tumefaciens* NT1 (*traR, tra::lacZ749*). They revealed the production of various patterns of the signal molecules, which are strain dependent. At least two signal molecules could be detected in most of the strains tested, and comparison of their relative mobilities suggested that they are homologs of *N*-octanoyl-DL-homoserine lactone (C_8-HSL) and *N*-decanoyl-DL-homoserine lactone (C_{10}-HSL).

Key words: Quorum sensing, *Methylobacterium*, *N*-acyl-homoserine lactone, thin-layer chromatography

Bacteria of the genus *Methylobacterium* (PPFMs, pink-pigmented facultative methylotrophic bacteria) are strictly aerobic, Gram-negative rods of *α-Proteobacteria*, capable of growth on C_1 compounds [12]. They are ubiquitous in nature and frequently associated with terrestrial and aquatic plants, colonizing roots and leaf surfaces and potentially dominating the phyllosphere bacterial population [4]. The knowledge on the *Methylobacterium*-plant interactions over the past two decades suggested interesting novel interactions between PPFMs and plants. Beneficial plant-growth promoting bacteria interact with plants through direct and indirect mechanisms. Direct mechanisms for the most part entail either providing the bacterial compounds that promote plant growth or facilitating the uptake of nutrients; for example, production of phytohormones [9] and siderophores [13]. The indirect effects occur through suppression of one or more phytopathogenic microorganisms through biocontrol [20] or induction of plant defense enzymes [14]. Beneficial effects of plant-*Methylobacterium* associations have been suggested to be due to production of phytohormones [22] and enzymes such as 1-aminocyclopropane-1-carboxylate (ACC) deaminase [15] that promote plant growth directly or indirectly, fixation of nitrogen [32], and systemic resistance induced against pathogens [16]. Since methylotrophic bacteria tend to form aggregates on the aerial parts of plants [31], the phenomenon of quorum sensing (QS), which affects the multicellular behavior of bacteria in a community, gains importance.

Quorum sensing is a mechanism involved in cell density-dependent bacterial communication that regulates the expression of specialized gene sets via secretion of specific low-molecular weight signal molecules in a given environment. At a threshold concentration, these signal molecules act as co-inducers to regulate transcription of target genes, consequently triggering specific behavioral responses in a bacterial population that presumably benefit the bacteria in the particular habitat [2, 21]. Most but not all of the quorum-sensing systems discovered involves *N*-acylated homoserine lactones (AHLs) as autoinducers. AHLs consist of a homoserine lactone moiety linked to an acyl side chain of variable length and substitution at the third carbon position that provide them with signal specificity. Much is known about the QS in a variety of heterotrophic proteobacteria; however, AHL production by methylotrophic bacteria has not yet been conclusively

*Corresponding author
Phone: 82-43-261-2561; Fax: 82-43-271-5921;
E-mail: tomsa@chungbuk.ac.kr

documented to date. A recent study revealed the presence of two QS systems in *M. extorquens* AM1, with production of a novel long-chained C14:2 (*N*-tetradecenoyl) AHL molecule [24].

The objectives of this study were to survey the *Methylobacterium* strains for the presence of AHL quorum signals and, if present, to characterize these molecules by thin-layer chromatography (TLC) coupled to bioassays. We further studied two *Methylobacterium* strains that had been proved to be plant-growth promoting, to study the production pattern of AHLs. Attempts were also made to confirm the presence of AHLs in the extracts through high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Methylobacterium strains CBMB20 (AY683045), CBMB110 (AY683046), CBMB120 (AY683047), CBMB130 (AY683048) tested for AHL production were isolated from rice and found to possess plant-growth promoting characteristics [28]. Strains from different species of *Methylobacterium*, screened for AHL production, along with their sources are listed in Table 1. Most strains were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany. Few strains from the Korean Agriculture Collection Centre (KACC) and the Korean Collection for Type Cultures (KCTC) were included. *Methylobacterium* strains were routinely grown in ammonium mineral salts (AMS) medium [35]. *Chromobacterium violaceum* CV026 [17] and *C. violaceum* ATCC12472 [18] were grown on Luria broth (LB) (Difco Laboratories, Detroit, Michigan, U.S.A.) at 30°C without any drugs. *Agrobacterium tumefaciens* strains were grown on AB minimal media with 0.2% mannitol (ABM) [3] at 28°C. *Pseudomonas putida* F117 (pRK-C12) [30] was grown on Luria Bertanii medium with 4 g of NaCl instead of 10 g of NaCl (MLB). The following concentrations of antibiotics were used: gentamycin, 20 μg/ml; kanamycin, 50 μg/ml; spectinomycin, 50 μg/ml; tetracycline, 4.5 μg/ml. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, BioShop Canada Inc., Burlington, ON, L7L4Y8) was supplemented in the medium for *A. tumefaciens* indicator bioassays. *N*-3-hexanoyl-DL-homoserine lactone (C_6-HSL), *N*-(3-oxo-hexanoyl)-DL-homoserine lactone (3-oxo-C_6-HSL), *N*-heptanoyl-DL-homoserine lactone (C_7-HSL), *N*-octanoyl-DL-homoserine lactone (C_8-HSL), and *N*-decanoyl-DL-homoserine lactone (C_{10}-HSL), *N*-dodecanoyl-DL-homoserine lactone (C_{12}-HSL), and *N*-tetradecanoyl-DL-homoserine lactone (C_{14}-HSL) were purchased from Fluka and Sigma (Sigma-Aldrich Co., St. Louis, MO, U.S.A.).

Diffusion Bioassays

One ml of overnight culture grown in ABM (*A. tumefaciens* NT1, *traR*, *tra::lacZ749* [25]) or LB (*C. violaceum*) was inoculated to 50 ml of the same medium and grown for 24 h at 30°C with shaking (120 rpm). The culture was then

Table 1. List of *Methylobacterium* strains used for testing AHL production and their sources.

Genus and species/strain	Accession No. from DSMZ/ KCTC, KACC collections	Source	Reference
Methylobacterium aquaticum	DSM 16371^T	Drinking water	Gallego *et al.* [8]
Methylobacterium dichloromethanicum	DSM 6343^T	Soil	Doronina *et al.* [7]
Methylobacterium extorquens	DSM 1337^T	Soil	Bousfield and Green [1]
Methylobacterium fujisawaense	KACC10744^T	Rhizoplane/*M. sativa*	Green *et al.* [11]
Methylobacterium hispanicum	DSM 16372^T	Drinking water	Gallego *et al.* [8]
Methylobacterium lusitanum	KCTC 12964^T	Sewage	Doronina *et al.* [6]
Methylobacterium mesophilicum	DSM 1708^T	Phylloplane/*L. perenne*	Green and Bousfield [10]
Methylobacterium organophilum	DSM 760^T	Sediment	Patt *et al.* [23]
Methylobacterium radiotolerans	DSM 1819^T	Rice grains	Green and Bousfield [10]
Methylobacterium rhodesianum	DSM 5687^T	Chlorinated water	Green *et al.* [11]
Methylobacterium rhodinum	DSM 2163^T	*Alnus* rhizosphere	Green and Bousfield [10]
Methylobacterium suomiense	KCTC 12963^T	Forest soil	Doronina *et al.* [6]
Methylobacterium thiocyanatum	DSM 11490^T	*A. aflatunense*/soil	Wood *et al.* [37]
Methylobacterium zatmanii	DSM 5688^T	Chlorinated water	Green *et al.* [11]
Methylobacterium sp. CBMB110	AY683046	Rice/leaf	This study
Methylobacterium sp. CBMB120	AY683047	Rhizosphere soil/rice	This study
Methylobacterium sp. CBMB130	AY683048	Rhizosphere soil/rice	This study
Methylobacterium sp. CBMB20	AY683045	Rice/stem	This study

^{T}Type strains collected from DSMZ, KCTC, or KACC collections. DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany; KACC, Korean Agriculture Collection Centre; KCTC, Korean Collection for Type Cultures. GenBank accession numbers for 16S ribosomal DNA sequences.

poured to 100 ml of molten ABM with X-Gal (60 μg/ml) for *A. tumefaciens* NT1 (1.12% agar) or molten LB for *C. violaceum* (0.8% agar) at 46°C and immediately poured over the surface of a 1.8% agar plate of the same medium. Fifty μl of supernatant was loaded into wells (6 mm) punched in the solidified top agar. *A. tumefaciens* KYC6, *C. violaceum* ATCC 31532, and C_{14}-HSL were used as positive controls for assays of *A. tumefaciens*, violacein induction, and inhibition in *C. violaceum*, respectively. Negative controls were the indicator strains themselves.

Relative Amounts of the AHLs Produced

The relative amount of the AHLs produced was estimated by a simple procedure outlined earlier by Dong *et al.* [5]. Briefly, agar slices of 1 cm width were cut from plates containing 20 ml of ABM agar supplemented with X-Gal (40 μg/ml) and used. The cell-free suspension of the test isolates was added to one end and *A. tumefaciens* NT1 (*traR, tra::lacZ749*) was spotted at progressively further distances from the loaded samples. The maximum distance of the indicator strain from the source, which resulted in a substrate conversion (blue spots), corresponded to the amount of AHLs that diffused from the tested cultures.

Extractions and Characterization of Signal (AHL) Molecules

For analytical TLC, AHLs were extracted from 20 ml of cultures grown in AMS media with 0.5% methanol. The supernatant of early-stationary-phase culture was extracted twice with equal volumes of acidified ethyl acetate (0.1 ml glacial acetic acid per liter ethylacetate). The organic phases were combined, dried over anhydrous magnesium sulfate, and evaporated to dryness by rotary evaporation at 37°C. The residue was resuspended in 300 μl of acidified ethyl acetate and stored at -20°C before use. Analytical TLC of the extracts was carried out as described by Shaw *et al.* [29]. Samples (3–6 μl) were applied to C_{18} reversed-phase TLC plates (20×20 cm, 200 μm layer; RP-18 F_{254s}, Merck, Germany) and chromatographed with a solvent system of methanol/water (60:40 v/v). After drying, the plates were overlaid with top agar containing the indicator strain, as described before, and incubated overnight at 30°C.

High-Performance Liquid Chromatography

Supernatants from 6 l of AMS-grown cultures of *Methylobacterium* strains CBMB120 and CBMB130 were extracted with dichloromethane, as described by McClean *et al.* [17]. Extracted AHLs were dried, redissolved in acetonitrile, and separated by reverse-phase C_{18} HPLC, using a column (19×100 mm; particle size 5 μm, Waters, Korea), which was eluted with a linear gradient of acetonitrile and water (20–100%) at a flow rate of 3 ml/min^{-1} and monitored at 210 nm. Both the retention time and spectral profiles were compared with those of synthetic AHL standards including C_6-HSL, C_8-HSL, and C_{10}-HSL. Fractions were collected and assayed for AHLs, using diffusion assay with *A. tumefaciens* NT1, as described above, and fluorescence assay with *P. putida* F117 (pRK-C12) [30]. The fluorescence activity for each fraction was assayed as described using a 100-μl volume [34]. Fluorescence was determined in a Victor1420 Multilabel Counter (Perkin-Elmer) at an excitation wavelength of 485 nm and a detection wavelength of 535 nm. Synthetic AHLs (C_{12}-HSL and C_{14}-HSL) were used as the positive control. OD_{600} was also measured. The relative fluorescence was expressed as the ratio of *gfp*535 to OD_{600}.

Kinetics of AHL Production

Methylobacterium strains, CBMB120 and CBMB130, and *M. extorquens* were precultured and grown in AMS medium with 0.5% methanol at 30°C, and growth was monitored by OD_{600} determinations. Samples were withdrawn at various times and cell-free supernatants were assayed for AHL contents by two independent methods; well diffusion assay and β-galactosidase assay. By well diffusion assay, AHL activity was measured as the ratio of the diameter of blue zone to the diameter of the well formed, when assayed with *A. tumefaciens* NT1 (*traR, tra::lacZ749*). For β-galactosidase assay, culture supernatants (200 μl) from various time points were added to 10 ml of *A. tumefaciens* NT1 (*traR, tra::lacZ749*) (OD_{600} of 0.7) and incubated for 6–8 h (120 rpm) at 30°C. After incubation, β-galactosidase was quantified as described by Miller [19]. The experiments were conducted in triplicate, and the values given indicate a mean of three individual experiments.

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) by using the general linear model Version 9.1; SAS Institute Inc., Cary, NC, U.S.A. Duncan's multiple range tests (DMRT) were used to determine differences between samples. Significance levels were within confidence limits of 0.05 or less. All treatments were done in triplicate.

RESULTS

AHL Signal Molecules in *Methylobacterium* Species

To investigate the presence of autoinducer signal molecules in *Methylobacterium,* we screened a total of 18 strains, which included 14 type strains of different species and 4 plant-associated *Methylobacterium* strains from our collection. We used preliminary plate diffusion assays for screening, with two different monitor systems that rely on induction of β-galactosidase activity in *A. tumefaciens* NT1 (*traR, tra::lacZ749*), and induction or inhibition of violacein in *C. violaceum* (CV026, CV12472). Culture supernatants from 12 strains out of 18 tested were found to

Table 2. Production of quorum-sensing signals and relative amounts of AHLs produced by different *Methylobacterium* species indicated by bioassays.

Methylobacterium strain	AHL production[a]	
	AHL activity[b]	Amount of AHLs[c]
Methylobacterium aquaticum	3.08±0.8	–
Methylobacterium dichloromethanicum	1.69±0.5	–
Methylobacterium extorquens	5.63±1.5	93.70±7.1
Methylobacterium hispanicum	5.30±1.4	49.30±0
Methylobacterium lusitanum	2.51±0.7	19.35±2.6
Methylobacterium rhodesianum	1.21±0.4	–
Methylobacterium rhodinum	3.22±0.8	15±0.5
Methylobacterium suomiense	5.87±1.5	315.18±53.4
Methylobacterium thiocyanatum	1.73±0.5	3.07±0.3
Methylobacterium zatmanii	2.27±0.6	–
Methylobacterium sp. CBMB120	5.59±1.5	165.58±22.6
Methylobacterium sp. CBMB130	5.53±1.4	130.44±17.8

[a]AHLs production was assayed using different indicators, and none of the strains tested elicited a response in *C. violaceum* indicator strains.
[b]AHL activity was measured as the ratio of the diameter of blue zone to the diameter of the well formed when assayed with *A. tumefaciens* NT1 (*traR, tra::lacZ749*).
[c]The amount of AHLs produced by *Methylobacterium* strains was calculated according to Dong *et al.* [5] and is expressed in ng.

induce β-galactosidase activity in the *A. tumefaciens* NT1 (*traR, tra::lacZ749*) monitoring system, resulting in blue zones. None of the strains tested elicited violacein production or inhibition in *C. violaceum*, whereas *C. violaceum* ATCC 31532 and C_{14}-HSL used as a positive control elicited the appropriate responses in these indicator strains. *M. suomiense* exhibited the highest AHL activity followed by *M. extorquens* (Table 2). The plant-associated *Methylobacterium* strains CBMB120 and CBMB130 exhibited higher AHL activity than other strains, and the strains CBMB20 and CBMB110 had no activity. The assay for relative amounts of AHLs revealed that the *Methylobacterium* strains CBMB120 and CBMB130 produced about 165.58 and 130.44 ng of AHLs, respectively. The results coincided with the results obtained by the diffusion assays with *M. suomiense*, producing a higher amount of AHLs than other strains. The other strains that produced relatively higher amounts of AHLs were *M. extorquens* and *M. hispanicum* (Table 2).

TLC Assays for AHL Complements and Sensitivity of Indicator Strains

TLC, coupled with a bioreporter, proves to be a simple and rapid method for assessing the minimum number of different AHLs produced and provides preliminary information concerning the nature of these compounds present in bacterial extracts [2]. Therefore, we assessed the extracts of culture supernatants of the bacterial strains by using

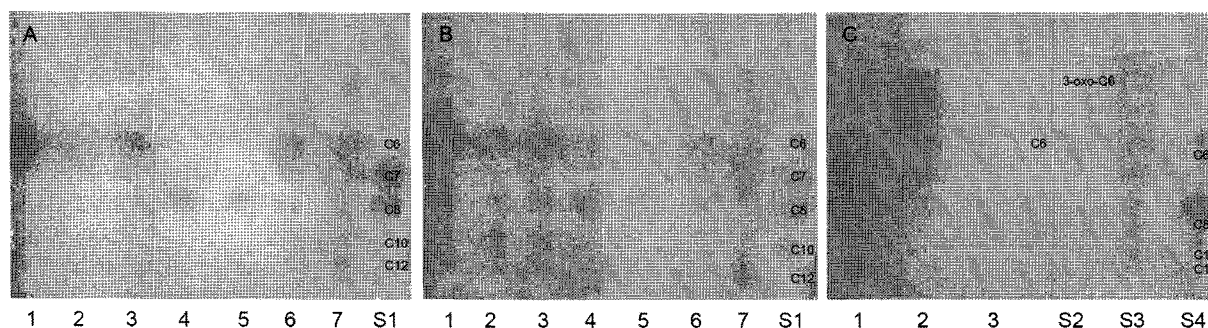

Fig. 1. TLC of AHL molecules in the ethylacetate extracts of *Methylobacterium*.
Samples were chromatographed on C_{18} reversed-phase thin-layer plates developed with methanol:water (60:40, vol/vol), and the spots were visualized with *A. tumefaciens* A136 (pCF218) (pCF372) (**A** and **C**) and *A. tumefaciens* NT1 (*traR, tra::lacZ749*) (**B**). Lanes 1 to 7 in **A** and **B** represent ethyl acetate extracts from 1-*Methylobacterium* sp. CBMB120; 2-*Methylobacterium* sp. CBMB130; 3-*M. suomiense*, 4-*M. extorquens*; 5-*M. hispanicum*, 6-*M. rhodinum* 7-*A. tumefaciens* KYC6. (**C**) Lanes 1, 2, and 3 represent the extracts from *A. tumefaciens* KYC6, *E. caratovora* subsp. *caratovora*, and *C. violaceum* 31532 that produce 3-oxo-C_8-HSL, 3-oxo-C_6-HSL, and C_6-HSL, respectively. S1, S2, S3, S4-Standards containing synthetic AHLs; the acyl chain lengths are indicated for each compound. S1-mixture of C_6 (1,000 μmol), C_7 (100 μmol), C_8 (1,000 μmol), C_{10} (1,000 μmol), and C_{12}-HSLs (10 mmol) S4-mixture of C_6, C_8, C_{10}, and C_{12}; S2-C_6-HSL (10 mmol); S3-3-oxo-C_6-HSL (1,000 μmol).

TLC coupled to a bioassay with *A. tumefaciens* NT1 (*traR, tra::lacZ749*). We also used another *traR*-based *A. tumefaciens* A136 with *traI-lacZ* fusion (pCF218) (pCF372) indicators for assessing TLC complements. Of the 18 strains tested, extracts from 7 strains were clearly shown to produce at least one signal molecule that was detectable by *A. tumefaciens* NT1 (*traR, tra::lacZ749*). Most of the *Methylobacterium* strains produced two signal molecules that migrated with R_f values similar to those of C_6-HSL (0.43) and C_8-HSL (0.24) that could be detected with *A. tumefaciens* NT1. However, none of the tested strains produced detectable levels of AHL molecules, when *C. violaceum* CV026 was used as the overlay. The plant-associated *Methylobacterium* strains, CBMB120 and CBMB130, and *M. suomiense* produced two additional molecules that co-migrated with the synthetic C_{10} (R_f - 0.08) and C_{12} HSLs (R_f - 0.01) (Fig. 1). *Methylobacterium* strain CBMB120 and *M. suomiense* produced two signal molecules detected by *A. tumefaciens* NT1 that migrated relatively fast, showing similar R_f values (0.52 and 0.65). The tentative structures for these molecules could not be assigned, since the standards used in this study did not match with them. The sensitivities of *A. tumefaciens* NT1 (*traR, tra::lacZ749*) and *A. tumefaciens* A136 (pCF218) (pCF372) indicators varied greatly in detecting the AHLs produced by *Methylobacterium*. For example, *A. tumefaciens* NT1 (*traR, tra::lacZ749*) detected two molecules from *M. extorquens* and *M. hispanicum*, whereas only one could be detected by *A. tumefaciens* A136 (pCF218) (pCF372) (Figs. 1A and 1B). However, *A. tumefaciens* A136 detected the synthetic standards C_6-, C_8- HSLs (Fig. 1C). Furthermore, no molecules could be detected with any of the indicator strains used, from the extracts of *M. rhodesianum, M. lusitanum, M. dichloromethanicum, M. thiocyanatum, M. zatmanii*, and *Methylobacterium* strains CBMB20 and CBMB110, even when their volume loaded was increased from 5 to 20 and 40 μl.

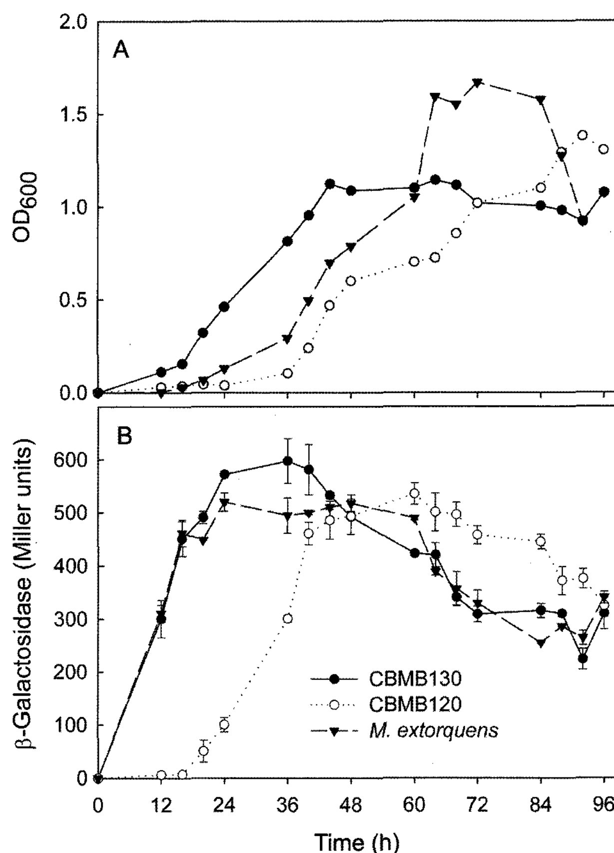

Fig. 2. AHL production and induction of *tra* gene activity in *A. tumefaciens* NT1 (*traR, tra::lacZ749*) by *Methylobacterium* as a function of time.
Growth was monitored by OD_{600} (**A**) and induction of β-galactosidase in *A. tumefaciens* NT1 (*traR, tra::lacZ749*) (**B**).

HPLC and Kinetics of AHL Molecules Produced by *Methylobacterium* spp.

Supernatants from *Methylobacterium* strains CBMB120 and CBMB130 produced AHL-inducing zones and possessed

Table 3. Results of the assays with *A. tumefaciens* NT1 (*traR, tra::lacZ749*) and *P. putida* F117 (pRK-C12) on the HPLC fractions of *Methylobacterium* strains CBMB120 and CBMB130.

CBMB120				CBMB130			
Fraction	R_t (min)	A136[a]	F117[b]	Fraction	R_t (min)	A136[a]	F117[b]
4	13.7	-	592.02	3	13.2	2.0	600.69
5	14.4	-	-	4	14.1	-	608.36
6	16.2	10.83	589.57	5	15.1	5.17	611.35
7	17.5	1.33	600.21	6	16.4	11.67	595.01
8	19.3	5.33	597.58	7	17.6	-	587.41
9	22.7	1.5	592.01	8	22.4	1.83	-
11	27	3.33	-	10	34.8	-	592.61
13	36	-	601.26	13	36.5	-	606.12

[a]The AHLs presence was assayed by the β-galactosidase zone formed in *A. tumefaciens* NT1 (*traR, tra::lacZ749*). AHL activity was measured as the ratio of the diameter of blue zone to the diameter of the well formed, when assayed with *A. tumefaciens* NT1 (*traR, tra::lacZ749*).
[b]The presence of AHLs is detected by the fluorescence induced in *P. putida* F117 (pRK-C12). The relative fluorescence is calculated as the (gfp535/OD_{600}) value.

β-galactosidase activity from 12 h onwards. The cell density was measured by concentration of AHLs that increased at a rate similar to growth (Fig. 2). Preparative reverse-phase HPLC was employed to confirm the presence of signal molecules in the extracts of *Methylobacterium* strains CBMB120 and CBMB130. The fractions collected at approximately 3-min intervals resulted in a total of 13 fractions for each strain. In CBMB120, five and six fractions elicited a positive response, respectively, with *A. tumefaciens* NT1 (*traR*, *tra::lacZ749*) and *P. putida* F117 (pRK-C12). In CBMB130, four fractions showed positive with the *A. tumefaciens* NT1 (*traR*, *tra::lacZ749*) monitoring system, whereas seven fractions induced fluorescence in *P. putida* F117 (pRK-C12) (Table 2). Also in the HPLC analyses, *Methylobacterium* strains CBMB120 and CBMB130 produced peaks that had retention times similar to C_8- (retention time, Rt: 36.6 min) and C_{10}-HSL (Rt: 37.59 min) synthetic standards.

DISCUSSION

Investigations with reporter assays indicated that production of compounds with AHL signaling activity is relatively common among the members of *Methylobacterium*. We used three different monitoring systems; the induction of β-galactosidase activity in *A. tumefaciens* NT1 (*traR*, *tra::lacZ749*), and induction or inhibition of violacein in *C. violaceum* CV026 or *C. violaceum* ATCC12472. The violacein in *C. violaceum* is induced by C_4-C_8 AHLs and inhibited by long-chain AHLs [2, 17]. In our study, no strains could induce the violacein production or inhibition in *C. violaceum*. *M. extorquens* AM1 produced the C_6- and C_8-HSL signal molecules detectable with *C. violaceum* CV026. Therefore, the failure to detect activities with *C. violaceum* in *Methylobacterium* strains indicates that these strains may contain AHL-mediated signaling systems, but produce signal molecules at below that of the levels detectable by the reporter [2]. However, it has been reported that the combined use of *C. violaceum* and *A. tumefaciens* systems serves as a good broad screening system [26, 29].

In our study, all the positive strains of *Methylobacterium* produced detectable levels of two signal molecules, when assessed by TLC with *A. tumefaciens* indicator strains. Although these two molecules had mobilities similar to those of C_6- and C_8-HSLs, they could not be detected with *C. violaceum* CV026 that readily responds to AHL molecules with C_4-C_8 chain lengths [2, 17]. The *A. tumefaciens traG::lacZ/traR* reporter detects 3-oxo- as well as 3-hydroxy-substituted HSL derivatives with acyl chain lengths from 4 to 12 carbons as well as signal molecules with acyl chain lengths longer than 12, and gives low responsiveness to unsubstituted acyl-HSLs. The 3-oxo and the 3-hydroxy derivatives of the same chain length migrate with indistinguishable mobilities, and the 3-oxo-C_8 and the 3-oxo-C_{10} co-migrate with C_6 and C_8 HSLs, respectively [29]. However, the possibility of these molecules to be 3-oxo derivatives could be ruled out, since the spots did not tail. Therefore, these molecules were tentatively identified to be 3-hydroxy derivatives of C_8 and C_{10} HSLs.

Some strains possessed additional active signals detected by *A. tumefaciens* NT1 (*traR*, *tra::lacZ749*). These additional signals may represent multiple quorum-sensing systems as in *Rhizobium meliloti* and *Pseudomonas aeruginosa*. Alternatively, they may be the products of a single acyl-HSL synthase that could produce several AHL derivatives [27, 36]. In *M. extorquens* AM1, two QS systems responsible for the production of short- and long-chain AHLs have been described [24], with the long-chain molecules being detected with *P. putida* F117 (pRK-C12), a *gfp*-based monitoring system. Our studies on diffusion assays and TLC analysis, which used only *A. tumefaciens* NT1 (*traR*, *tra::lacZ749*), proved that the production of AHL-like signal molecules is more common among *Methylobacterium*.

We included the *P. putida* F117 (pRK-C12) indicator that could detect long-chain AHL molecules from C_8-C_{14} and their 3-oxo-substituents for assessing the positive fractions of HPLC from the plant-associated *Methylobacterium* strains CBMB120 and CBMB130 [30, 34]. The presence of nonpolar compounds could not be detected in a 60:40 (v/v) methanol: water solvent system with TLC using *P. putida* F117 (pRK-C12) [24]. Hence, we assessed the fractions of the extracts from *Methylobacterium* strains CBMB120 and CBMB130, which were purified by HPLC, for the induction of fluorescence in *P. putida* F117 (pRK-C12) by plate assays. The presence of positive fractions that elicited fluorescence in *P. putida* F117 (pRK-C12) revealed that the *Methylobacterium* strains CBMB120 and CBMB130 used in this study may also produce some novel molecules in addition to those identified by TLC. Taken together with our results from TLC and HPLC analyses, it is consistent that *Methylobacterium* strains CBMB120 and CBMB130 produce at least three types of AHL-like molecules, and that the production of AHL-like molecules appears to be widespread among genetically distinct *Methylobacterium* strains. The results from AHL measurements by well diffusion and β-galactosidase assays with three *Methylobacterium* strains revealed that synthesis of AHL in *Methylobacterium* is constitutive. Our results are consistent with previous reports on AHL production by some *Enterobacteriaceae* and *Yersinia enterocolitica* [26, 33].

In conclusion, this study proves for the first time that the occurrence of quorum-sensing systems in *Methylobacterium* is widespread. Further investigations on the identification of the chemicals produced and the genes involved in this global regulatory system in *Methylobacterium* may help to understand their unique methylotrophic characteristics.

Acknowledgments

We thank Robert J. C. McLean, Texas State University-San Marcos, U.S.A.; Sylvia Jafra, Plant Research International, Wageningen, for providing the indicator strains used in this study. M. M. and S. P. are indebted to the Korea Research Foundation, Republic of Korea, for a position as invited foreign scientist and PhD research grants, respectively. We are grateful for the financial supports rendered through Agricultural Research Promotion Centre, Republic of Korea is acknowledged. We also thank Miguel Camara for contributing to helpful discussions during the experimentation.

References

1. Bousfield, I. J. and P. N. Green. 1985. Reclassification of bacteria of the genus *Protomonas* Urakami and Komagata 1984 in the genus *Methylobacterium* (Patt, Cole, and Hanson) emend. Green and Bousfield 1983. *Int. J. Syst. Bacteriol.* **35:** 209.
2. Cha, C., P. Gao, Y. C. Chen, P. D. Shaw, and S. K. Farrand. 1998. Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria. *Mol. Plant-Microbe Interact.* **11:** 1119–1129.
3. Chilton, M. D., T. C. Currier, S. K. Farrand, A. J. Bendich, M. P. Gordon, and E. W. Nester. 1974. *Agrobacterium tumefaciens* and P58 bacteriophage DNA not detected in crown gall tumour DNA. *Proc. Natl. Acad. Sci. USA* **71:** 3672–3676.
4. Corpe, W. A. 1985. A method for detecting methylotrophic bacteria on solid surfaces. *J. Microbiol. Methods* **3:** 215–221.
5. Dong, Y. H., J. L. Xu, X. Z. Li, and L. H. Zhang. 2001. Aii, an enzyme that inactivates the acylhomoserine lactone quorum sensing signal and attenuates the virulence of *Erwinia carotovora*. *Proc. Natl. Acad. Sci. USA* **97:** 3526–3531.
6. Doronina, N. V., Y. A. Trotsenko, B. B. Kuznetsov, T. P. Tourova, and M. S. Salkinoja-Salonen. 2002. *Methylobacterium suomiense* sp. nov. and *Methylobacterium lusitanum* sp. nov., aerobic, pink-pigmented, facultatively methylotrophic bacteria. *Int. J. Syst. Evol. Microbiol.* **52:** 773–776.
7. Doronina, N. V., Y. A. Trotsenko, T. P. Tourova, B. B. Kuznetsov, and T. Leisinger. 2000. *Methylopila helvetica* sp. nov. and *Methylobacterium dichloromethanicum* sp. nov. - novel aerobic facultatively methylotrophic bacteria utilizing dichloromethane. *Syst. Appl. Microbiol.* **23:** 210–218.
8. Gallego, V., M. T. Garcia, and A. Ventosa. 2005. *Methylobacterium hispanicum* sp. nov. and *Methylobacterium aquaticum* sp. nov., isolated from drinking water. *Int. J. Syst. Evol. Microbiol.* **55:** 281–287.
9. Glick, B. R. 1995. The enhancement of plant growth by freeliving bacteria. *Can. J. Microbiol.* **41:** 109–117.
10. Green, P. N. and I. J. Bousfield. 1983. Emendation of *Methylobacterium* Patt, Cole, and Hanson 1976; *Methylobacterium rhodinum* (Heumann 1962) comb. nov. corrig.; *Methylobacterium radiotolerans* (Ito & Iizuka 1971) comb. nov., corrig.; and *Methylobacterium mesophilicum* (Austin & Goodfellow 1979) comb. nov. *Int. J. Syst. Bacteriol.* **33:** 875–877.
11. Green, P. N., I. J. Bousfield, and D. Hood. 1988. Three new *Methylobacterium* species: *M. rhodesianum* sp. nov., *M. zatmanii* sp. nov., and *M. fujisawaense* sp. nov. *Int. J. Syst. Bacteriol.* **38:** 124–127.
12. Green, P. N. 1992. The genus *Methylobacterium*, pp. 2342–2349. *In* Balows, A., Trüper, H. G., Dworkin, M., Harder, W. and Schleifer, K. H. (eds.), *The Prokaryotes*, 2nd Ed., vol. III. Springer-Verlag, New York, U.S.A.
13. Katiyar, V. and R. Goel. 2004. Improved plant growth from seed bacterization using siderophore overproducing cold resistant mutant of *Pseudomonas fluorescens*. *J. Microbiol. Biotechnol.* **14:** 653–657.
14. Lee, H. Y., K. H. Park, J. H. Shim, R. D. Park, Y. W. Kim, J. Y. Cho, H. B. Hoon, Y. C. Kim, G. S. Cha, H. B. Krishnan, and K. Y. Kim. 2005. Quantitative changes of plant defense enzymes in biocontrol of pepper (*Capsicium annuum L.*) late blight by antagonistic *Bacillus subtilis* HJ927. *J. Microbiol. Biotechnol.* **15:** 1073–1079.
15. Madhaiyan, M., S. Poonguzhali, J. H. Ryu, and T. M. Sa. 2006. Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. *Planta* **224:** 268–278.
16. Madhaiyan, M., S. Poonguzhali, M. Senthilkumar, S. Seshadri, H. Y. Chung, J. C. Yang, S. P. Sundaram, and T. M. Sa. 2004. Growth promotion and induction of systemic resistance in rice cultivar Co-47 (*Oryza sativa* L.) by *Methylobacterium* spp. *Bot. Bull. Acad. Sin.* **45:** 315–324.
17. McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, J. H. Lamb, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams. 1997. Quorum sensing and *Chromobacterium violaceum*: Exploitation of violacein production and inhibition for the detection of *N*-acyl homoserine lactones. *Microbiology* **143:** 3703–3711.
18. McLean, R. J. C., L. S. Pierson, and C. Fuqua. 2004. A simple screening protocol for the identification of quorum signal antagonists. *J. Microbiol. Methods* **58:** 351–360.
19. Miller, J. H. 1972. Assay of β-galactosidase, pp. 352–355. *In*: *Experiments of Molecular Genetics*. Cold Spring Harbour Laboratory Press, New York.
20. Nautiyal, C. S., S. Mehta, and H. B. Singh. 2006. Biological control and plant-growth promotion by *Bacillus* strains from milk. *J. Microbiol. Biotechnol.* **16:** 184–192.
21. Newton, J. A. and R. G. Fray. 2004. Integration of environmental and host-derived signals with quorum sensing during plant-microbe interactions. *Cell Microbiol.* **6:** 213–224.
22. Omer, Z. S., R. Tombolini, A. Broberg, and B. Gerhardson. 2004. Indole-3-acetic acid production by pink-pigmented facultative methylotrophic bacteria. *Plant Growth Regul.* **43:** 93–96.

23. Patt, T. E., G. C. Cole, and R. S. Hanson. 1976. *Methylobacterium*, a new genus of facultatively methylotrophic bacteria. *Int. J. Syst. Bacteriol.* **26:** 226–229.
24. Penalver, C. G. N., D. Morin, F. Cantet, O. Saurel, A. Milon, and J. A. Vorholt. 2006. *Methylobacterium extorquens* AM1 produces a novel type of acyl-homoserine lactone with a double unsaturated side chain under methylotrophic growth conditions. *FEBS Lett.* **580:** 561–567.
25. Piper, K. R., B. V. S. Bodman, and S. K. Farrand. 1993. Conjugation factor of *Agrobacterium tumefaciens* Ti plasmid transfer by autoinduction. *Nature* **362:** 448–450.
26. Ravn, L., A. B. Christensen, S. Molin, M. Givskov, and L. Gram. 2001. Methods for identifying and quantifying acylated homoserine lactones produced by Gram-negative bacteria and their application in studies of AHL-production kinetics. *J. Microbiol. Methods* **44:** 239–251.
27. Rosemeyer, V., J. Michiels, C. Verreth, and J. Vanderleyden. 1998. *luxI* and *luxR*-homologous genes of *Rhizobium etli* CNPAF512 contribute to synthesis of autoinducer molecules and nodulation of *Phaseolus vulgaris*. *J. Bacteriol.* **180:** 815–821.
28. Ryu, J. H., M. Madhaiyan, S. Poonguzhali, W. J. Yim, P. Indiragandhi, K. A. Kim, R. Anandham, J. C. Yun, K. H. Kim, and T. M. Sa. 2006. Plant growth substances produced by *Methylobacterium* spp. and their effect on tomato (*Lycopersicon esculentum* L.) and red pepper (*Capsicum annuum* L.) growth. *J. Microbiol. Biotechnol.* **16:** 1622–1628.
29. Shaw, P. D., G. Ping, S. L. Daly, C. Cha, J. E. Cronan, K. L. Rinehart, and S. K. Farrand. 1997. Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc. Natl. Acad. Sci. USA* **94:** 6036–6041.
30. Steidle, A., K. Sigl, R. Schuhegger, A. Ihring, M. Schmid, S. Gantner, M. Stoffels, K. Riedel, M. Givskov, A. Hartmann, C. Langebartels, and L. Eberl. 2001. Visualization of *N*-acylhomoserine lactone-mediated cell-cell communication between bacteria colonizing the tomato rhizosphere. *Appl. Environ. Microbiol.* **67:** 5761–5770.
31. Sy, A., A. C. J. Timmers, C. Knief, and J. A. Vorholt. 2005. Methylotrophic metabolism is advantageous for *Methylobacterium extorquens* during colonization of *Medicago truncatula* under competitive conditions. *Appl. Environ. Microbiol.* **71:** 7245–7252.
32. Sy, A., E. Girud, P. Jourand, N. Garcia, A. Willems, P. De Lajudie, Y. Prin, M. Neyra, M. Gills, B. M. Catherine, and B. Dreyfus. 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix atmospheric nitrogen in symbiosis with legumes. *J. Bacteriol.* **183:** 214–220.
33. Throup, J. P., M. Camara, G. S. Briggs, M. K. Winson, S. R. Chhabra, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart. 1995. Characterization of the *yen*I/*yen*R locus from *Yersinia enterocolitica* mediating the synthesis of two *N*-acylhomoserine lactone signal molecules. *Mol. Microbiol.* **17:** 345–356.
34. Wagner-Döbler, I., V. Thiel, L. Eberl, M. Allgaier, A. Bodor, S. Meyer, S. Ebner, A. Hennig, R. Pukall, and S. Schulz. 2005. Discovery of complex mixtures of novel long-chain quorum sensing signals in free-living and host associated marine *Alphaproteobacteria*. *Chembiochem* **6:** 2195–2206.
35. Whittenbury, R., S. L. Davies, and J. F. Wilkinson.1970. Enrichment, isolation and some properties of methane-utilizing bacteria. *J. Gen. Microbiol.* **61:** 205–218.
36. Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. C. Salmond, B. W. Bycroft, A. Lazdunski, G. S. A. B. Stewart, and P. Williams. 1995. Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **92:** 9427–9431.
37. Wood, A. P., D. P. Kelly, I. R. McDonald, S. L. Jordan, T. D. Morgan, S. Khan, J. C. Murrell, and E. Borodina. 1998. A novel pink-pigmented facultative methylotroph, *Methylobacterium thiocyanatum* sp. nov., capable of growth on thiocyanate or cyanate as sole nitrogen sources. *Arch. Microbiol.* **169:** 148–158.