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Characterization of Quorum-Sensing Signaling Molecules Produced by *Burkholderia cepacia* G4

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Abstract In many Gram-negative bacteria, autoinducers, such as *N*-acyl-L-homoserine lactone (acyl-HSL) and its derivative molecules, mediate the cell-density-dependent expression of certain operons. The current study identified the autoinducers produced by *Burkholderia cepacia* G4, a trichloroethylene-degrading lagoon isolate, using TLC bioassays with *Agrobacterium tumefaciens* NT1(pDCI41E33) and *Chromobacterium violaceum* CV026, and a GC-MS analysis. The R_f and R_t values and mass spectra were compared with those of synthetic compounds. Based on the analyses, it was confirmed that G4 produces *N*-hexanoyl (C6)-, *N*-octanoyl (C8)-, *N*-decanoyl (C10)-, *N*-dodecanoyl (C12)-HSL, and an unknown active species. The integration of the GC peak areas exhibited a ratio of C8-HSL:C10-HSL:C12-HSL at 3:17:1 with C6-HSL and C10-HSL production at trace and micromolar levels, respectively, in the culture supernatants. Mutants partially defective in producing acyl-HSLs were also partially defective in the biosynthesis of an antibiotic substance. These results indicate that the autoinducer-dependent gene regulation in G4 is dissimilar to the clinical *B. cepacia* strains isolated from patients with cystic fibrosis.

Key words: *Burkholderia cepacia*, quorum sensing, *N*-acyl-L-homoserine lactone, cystic fibrosis, autoinducer

In bacteria, some gene sets are expressed when the cell density reaches a critical level. The bacteria monitor their own population density by sensing the concentration of membrane-diffusible small molecules that they themselves produce. These molecules are called autoinducers. These autoinducers bind to transcriptional activator(s) to control the expression of certain gene sets in response to cell growth. This type of signaling system is referred to as quorum sensing [8].

Quorum sensing was first described in the marine symbiotic bacterium *Vibrio fischeri* [22]. This bacterium expresses genes encoding bioluminescence at a threshold population density. At high cell densities, the diffusible *V. fischeri* autoinducers, VAI, accumulate in a confined environment, such as in specialized organs in fish, and bind to a transcriptional activator, LuxR. This in turn activates the transcription of the *lux* operon including the *luxI* gene, whose gene product, LuxI, is an autoinducer synthase (for reviews, see references [7, 8]). The chemical structure of VAI is *N*-3-(oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) [5]. Over the past decade, many Gram-negative bacteria, including plant and animal pathogens and soil isolates, have been shown to exploit LuxR-LuxI type quorum-sensing systems to control various phenotypes with *N*-acyl-L-HSLs (acyl-HSLs) as autoinducers. Such acyl-HSLs produced by various bacteria can include variations in length, substitution at the 3-carbon position, and the level of saturation in the acyl chain. In addition, many bacteria are known to produce multiple quorum-sensing signals that are formed either by a single acyl-HSL synthase or by multiple quorum-sensing systems. The targets controlled by quorum sensing include the production of virulence factors in some pathogens, bacterial motility, the development of biofilms in *Pseudomonas aeruginosa*, the conjugal transfer of Ti plasmids in *Agrobacterium tumefaciens*, and the production of secondary metabolites such as antibiotics and pigments [7, 8, 11, 14, 15, 24, 29, 32].

Burkholderia cepacia belonging to the β-subclass of Proteobacteria was originally described as a phytopathogen of onion [1]. The microorganism, found in soil and moist environments, is well known for its metabolic versatility, plus it even degrades various toxic and recalcitrant chemicals

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[4, 16, 23]. Other strains are also very effective in controlling plant pathogenic soil fungi and nematodes [17, 18, 37]. Thus, this organism is considered as an important biological agent for both biodegradation and agriculture [12]. In contrast, it has also emerged as an opportunistic human pathogen, especially as the causative agent in respiratory infections of cystic fibrosis [10, 12]. Despite its wide distribution in various habitats, studies on quorum sensing in *B. cepacia* have so far been limited to clinical strains. For instance, a recent study showed that the clinical isolate, *B. cepacia* K56-2, possesses quorum sensing with the LuxRI homologs CepRI and *N*-octanoyl-L-HSL (C8-HSL) as the autoinducer in the regulation of extracellular virulence factors [19]. This study examined the types and role of acyl-HSLs from the toluene-degrading lagoon isolate *B. cepacia* G4. The results showed that there is strain variation in quorum sensing in *B. cepacia* strains, depending on the origin of isolation.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions

B. cepacia G4 was originally isolated from a waste treatment lagoon, based on its ability to degrade trichloroethylene and toluene via a toluene *ortho*-monooxygenase (TOM) [23, 27]. The reporter strains used for the bioassays to detect the acyl-HSLs were *A. tumefaciens* NT1(pDCI41E33) [26] and *Chromobacterium violaceum* CV026 [20]. The former strain is a *tra* reporter containing a fused β-galactosidase gene, whereas the latter is used to detect the development of purple pigment violacein in bioassays. All strains were routinely maintained and cultured in a Luria-Bertani (LB) medium with appropriate antibiotics. For the bioassays, strain NT1(pDCI41E33) was cultured in a minimal salts basal (MSB) medium [31] containing 0.1% mannitol and cabenicillin, for 3 days at 28°C. Strain CV026 was cultured overnight in an LB liquid medium at 28°C. The reporter cells were collected by centrifugation and stored at –72°C until use. G4 was grown in LB, Potato dextrose (Difco Laboratories, MD, U.S.A.), or MSB media with an appropriate carbon source in a shaking incubator at 28°C with reciprocal shaking (180 rpm). The medium volumes were 100 ml in 500-ml Erlenmeyer flasks. For the identification and detection of the acyl-HSL molecules produced by G4, the cells were grown in MSB containing 0.5% ethanol as the carbon/energy source for 3 days. For the antibiotic assays, *Micrococcus luteus* KCTC1054 was used as the test strain and grown in a Nutrient broth (Difco Laboratories, MO, U.S.A.). *Escherichia coli* S17-1(pJFF350) [6] was used for the Omegon-Km mutagenesis and grown in LB containing kanamycin. The antibiotics were used at 25 μg/ml for kanamycin, 50 μg/ml for ampicillin, and 50 μg/ml for cabenicillin.

Chemicals

The acyl-HSLs were synthesized as described previously by Zhang *et al.* [38]. The chemicals used for the synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Autoinducer Bioassays

The petri dish and thin layer chromatography (TLC) assays were carried out as previously described [2, 26] with the following modifications: For the petri dish assays, indicated amounts of culture supernatants were loaded on paper discs (dia. 0.8 cm, Toyo Roshi Kaisha Ltd, Japan), then the discs were dried before being transferred onto the surface of a soft agar overlay which contained agar (0.8%), mannitol (0.1%), X-gal (0.1 mg/ml), and the indicator, NT1(pDCI41E33). For the TLC analysis, the culture supernatants were extracted with ethyl acetate and then concentrated on a rotary evaporator at 35°C, as described previously [3]. The extracts were dissolved in methanol at 1,000-fold. The culture extracts were applied to C18-reverse-phase TLC plates (0.2 mm thickness, E. Merck, Germany), then the TLC was developed in a closed chamber with a solution of methanol and water (7:3, v/v). The plates were dried and bioassays were conducted with NT1(pDCI41E33) or CV026, as previously described [2, 26], to identify the autoinducer spots.

Siderophore Production, Protease, and Lipase Assays

The siderophore activity was measured on a CAS (chrome azurol S, Aldrich) agar supplemented with 0.1% mannitol [25]. The production of siderophores by microorganisms results in a blue-to-orange color change in the zones surrounding the colonies. Protease assays were carried out on a dialyzed brain-heart infusion (D-BHI) and skimmed milk agar, as previously described by Sokol *et al.* [30]. The lipase activity was screened on a lipase indicator plate, as previously described [13].

Assays for Antibiotic Production

Indicated amounts of culture supernatants were loaded on paper discs. The paper discs were dried in air, and then transferred onto the surface of the top nutrient agar overlay (0.8% agar) containing the indicator, *M. luteus* KCTC1054. The KCTC1054 cells for the bioassays were prepared as follows: The cells were grown overnight at 28°C with vigorous shaking (180 rpm), and then a 100-fold diluted original culture broth was added to the top agar. Those culture supernatants with antibacterial activity showed clear zones around the paper discs after 48 h of incubation at 28°C.

GC-MS Analysis

Gas chromatography-mass spectrometry (GC-MS) was performed using a Hewlett-Packard model 6890 Plus GC

(U.S.A.) equipped with a Hewlett-Packard Ultra-1 capillary column (0.2 mm×25 m; film thickness 0.33 μm). The column temperature was initially kept at 70°C for 5 min and then programmed from 70 to 300°C at 10°C/min with a helium flow of 25 cm/sec. The temperatures of the injection port, transfer line, and detector were 280, 280, and 250°C, respectively. Samples (1 μl each) prepared as described above in 'Autoinducer Bioassays' were injected in a splitless mode, and the mass spectra were obtained using a Hewlett-Packard model HP5973 mass selective detection with electric impact ionization (70 eV). For the GC-MS analysis, prior to extraction, dibutyl phthalate was added as the internal standard to the culture supernatant at a final concentration of 4.0 μM to evaluate the efficiency of extraction. The amounts of *N*-decanoyl-L-HSL (C10-HSL) produced by G4 were calculated from the standard curve of synthetic C10-HSL.

Mutagenesis and Isolation of Autoinducer-Negative G4 Mutants

The introduction and subsequent transposition of Omegon-Km from pJFF350 into the genome of G4 was carried out by mating G4 and *E. coli* S17-1(pJFF350) on a nitrocellulose filter, as described by Shields *et al.* [28]. The Omegon-Km-derived mutants of G4 were selectively grown on an LB agar containing ampicillin and kanamycin. To isolate the autoinducer-negative mutants, each colony was toothpicked onto an MSB agar containing mannitol (0.1%), X-gal (0.1 mg/ml), and NT1(pDCI41E33) on the surface. The autoinducer-negative strains were defective in producing the blue zones formed by the degradation of X-gal. The G4 mutants were further isolated from NT1(pDCI41E33) by streaking on an MSB agar containing 0.1% sodium benzoate, which cannot support the growth of the latter strain.

RESULTS

Effect of Growth Conditions on Autoinducer Production by G4

Although the G4 strain is well known for its ability to degrade toluene and trichloroethylene using a TOM pathway [23, 27], little is known about the production of compounds with acyl-HSL signaling activity. Our primary study showed that G4 produces a large quantity of acyl-HSL molecules. In order to determine the relationship between the cell culture conditions and the autoinduction, the levels of autoinducers produced in different media were measured at 12-h intervals of culture time. The G4 grown in MSB with carbon and energy sources of either ethanol, Na-phthalate, glucose, Na-benzoate, mannitol, Na-succinate, or ethyl acetate at 0.1% supported the cell-density-dependent production of autoinducers. Among the carbon and energy sources, the maximal production of autoinducer was obtained in ethanol. A comparison of the patterns of autoinducer production by G4 was made, when it was grown in complex and minimal salts media with 0.5% ethanol. As shown in Fig. 1, in both LB and MSB, the production of autoinducers was highly related to the cell growth and was maximally attained in the late log phase, although the production levels were different in two different media. Interestingly, the autoinducers produced in LB rapidly degraded after the early stationary phase, and

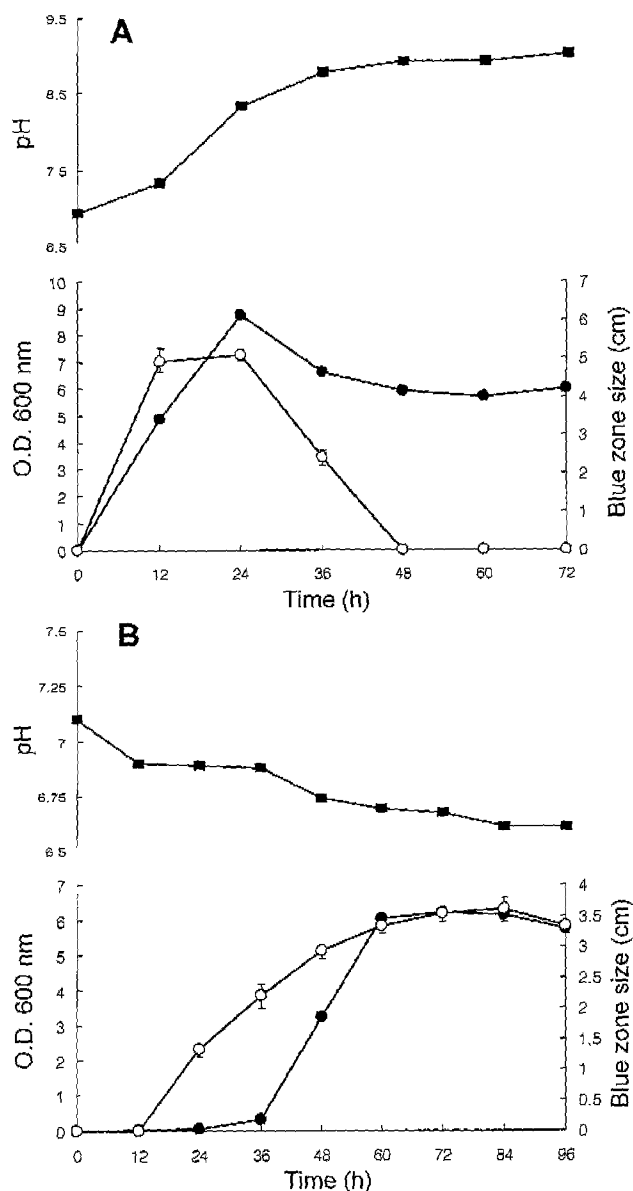

Fig. 1. Profile of autoinducer production by *B. cepacia* G4 when growing in LB (A) and MSB medium with 0.5% ethanol (B).

The cell growth was measured at an optical density of 600 nm (solid circles). The supernatants (each 30 μl) were used for autoinducer bioassays with *A. tumefaciens* NT1(pDCI41E33) (open circles), as described in Materials and Methods.

Table 1. TLC and GC-MS properties of synthetic acyl-HSLs.

Compound[a]	TLC[b] R_f	GC-MS data[c]	
		R_t (min)	Ion fragment *m/z* values (% relative intensity)
C4	0.67	17.42	171 (M^+, 5.84), 153 (4.65), 143 (75.49), 125 (7.00), 113 (7.00), 101 (9.34), 85 (9.73), 71 (58.75), 57 (90.27), 43 (100)
C6	0.52	19.79	199 (M^+, 1.59), 170 (3.57), 156 (10.32), 143 (100), 125 (21.43), 99 (23.81), 83 (20.63), 71 (30.16), 57 (64.68), 43 (68.25)
C8	0.32	22.04	227 (M^+, 2.77), 198 (1.58), 184 (1.98), 170 (2.37), 156 (15.42), 143 (100), 125 (19.37), 101 (15.02), 83 (15.81), 57 (85.77), 43 (26.09)
C10	0.16	24.14	255 (M^+, 4.33), 212 (2.36), 198 (1.97), 170 (1.97), 156 (17.32), 143 (100), 125 (17.72), 102 (19.69), 83 (14.96), 71 (11.81), 57 (47.24), 43 (27.56)
C12	0.06	26.02	283 (M^+, 7.14), 240 (1.59), 212 (1.98), 198 (2.38), 183 (2.38), 170 (1.98), 156 (21.43), 143 (100), 125 (17.46), 102 (19.84), 83 (17.06), 71 (11.90), 57 (47.62), 43 (25.40)

[a]Abbreviations for chemicals: C4, *N*-butanoyl-L-HSL; C6, *N*-hexanoyl-L-HSL; C8, *N*-octanoyl-L-HSL; C10, *N*-decanoyl-L-HSL; C12, *N*-dodecanoyl-L-HSL.
[b]TLC was developed with a mixture of methanol and water (7:3, v/v) on a C18-reverse-phase plate, as described in Materials and Methods.
[c]The GC-MS conditions are described in Materials and Methods.

became undetectable after 48 h. It is known that acyl-HSLs are chemically stable at acidic and neutral pH, and the lactone ring of HSL is hydrolyzed at alkaline pH in aqueous solutions (e.g., $t_{1/2}<3$ h at pH 8.0) [34]. In addition, it has not been reported that acyl-HSL-producing bacteria have an enzyme to degrade the signal(s). The culture conditions in MSB were maintained at neutral and slightly acidic pH during growth, whereas in the LB medium, they became alkaline with increasing culture time (Fig. 1). Therefore, the pH would appear to be an important factor that controls the half-life of the autoinducers produced by G4 in the culture media.

Detection of Autoinducers by TLC Bioassays

The TLC bioassay is a convenient method for the preliminary identification of acyl-HSL molecules. In TLC bioassays, the ethyl acetate extracts of the culture supernatants are first developed on a C18-reverse-phase thin-layer gel with a mixture of methanol and water. The autoinducers migrate on the gel according to their solubility in the developing solvent. The autoinducer spots are detected using a reporter strain that expresses an easily detectable trait in response to exogenous autoinducers. Among acyl-HSL bioreporters, the *traG::lacZ* fusion reporter of *A. tumefaciens* NT1(pDCI41E33) is one of the most sensitive and versatile detectors [26]. However, the bioreporter showed no activity towards *N*-butanoyl-L-HSL (C4-HSL). Another bioreporter, the pigment-negative mutant of *C. violaceum* CV026, produces a violet-colored violacein with exogenous autoinducers and has been used to detect short chain acyl-HSLs [2, 20]. This sensor exhibited the highest sensitivity to C6-HSL. These two bioreporters were used in the TLC bioassays to detect the acyl-HSL molecules produced by G4.

The initial results, such as the R_f values and the absence of the spots tailing [26], suggested that the acyl-HSLs produced by G4 were unsubstituted acyl-HSLs. In order to compare the properties between authentic and biological compounds, acyl-HSLs containing varying acyl side chains were synthesized. The GC-MS and TLC properties of the synthetic acyl-HSLs are listed in Table 1. In the TLC bioassays with NT1(pDCI41E33), three spots chromatographed with *N*-hexanoyl-L-HSL (C6-HSL), *N*-octanoyl-L-HSL (C8-

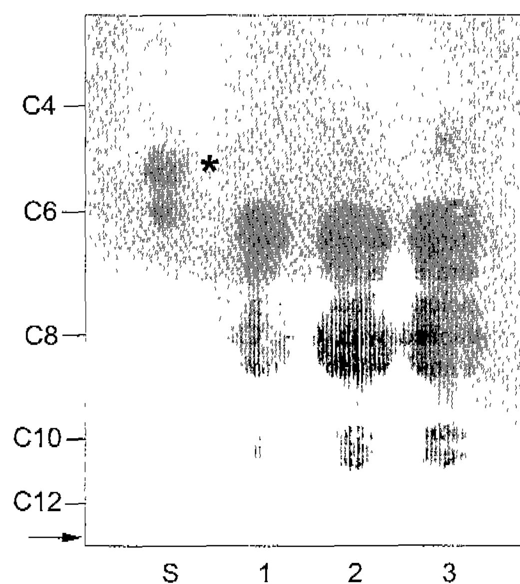

Fig. 2. TLC bioassay of acyl-HSL molecules with *A. tumefaciens* NT1(pDCI41E33).
The abbreviations for the synthetic standards are given in Table 1. C4 shows the position at which authentic C4-HSL migrates, and this position was deduced from Fig. 3. The asterisk marks an unknown active component that was a contaminant originating from the C4-HSL standard, as also detected in a previous report [26]. The sample was from an ethyl acetate extract of the culture supernatant of *B. cepacia* G4: lane S, authentic acyl-HSLs; lane 1, 1 μl; lane 2, 5 μl; lane 3, 10 μl. The arrow shows the origin of the spots.

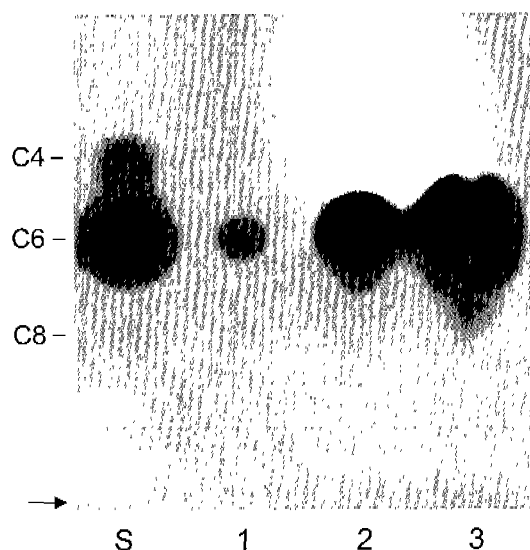

Fig. 3. TLC bioassay of acyl-HSL molecules with *C. violaceum* CV026.

The abbreviations for the standards are the same as in Table 1. The sample was from the ethyl acetate extract used in Fig. 2. Lane S, authentic acyl-HSLs; lane 1, 0.5 μl; lane 2, 5 μl; lane 3, 25 μl. The arrow shows the origin of the spots.

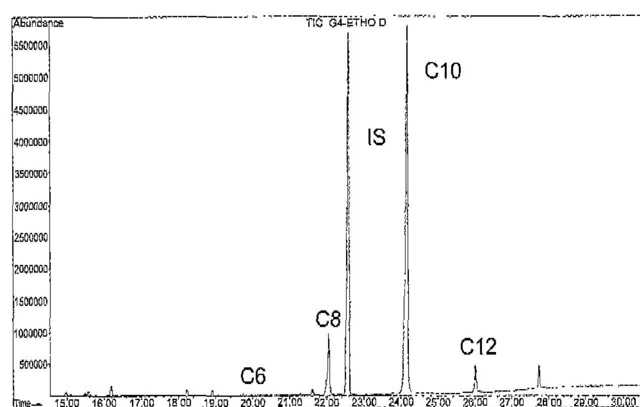

Fig. 4. GC profile of ethyl acetate extract. The sample was the same as that used in Fig. 2.

The abbreviations for each peak are indicated in Table 1. IS stands for dibutyl phthalate, used as the internal standard. The peak with a retention time at 27.75 min did not possess the possible ion fragments for acyl-HSL, such as an *m/z* 143, but had the ion fragments for a GC column material.

HSL), and C10-HSL were detected, along with one spot with the fastest mobility (Fig. 2). The fast migrating spot did not comigrate with an unknown active compound (*) found from the standard mixture. At a higher sample load, there was another spot comigrating with *N*-dodecanoyl-L-HSL (C12-HSL). In addition, another TLC bioassay was carried out with CV026 to detect C4-HSL. As originally determined [20], CV026 was not active with the acyl-HSLs containing acyl side chains longer than 10, yet were active with shorter acyl-HSLs, as shown in Fig. 3. Even at a larger sample load, no C4-HSL was detected in the culture extract of G4. From these TLC analyses, it was concluded that G4 produces autoinducers that chromatographed with C6-, C8-, C10-, and C12-HSLs, and another unidentifiable autoinducer.

Quantification of Acyl-HSL Molecules Produced by G4 with GC-MS Analysis

Since the biosensor strains used in this study have different sensitivities towards acyl-HSLs, the density or the size of the spots from the TLC bioassays could not be used to calculate the relative amounts of acyl-HSLs produced [26]. Therefore, to determine the amounts of acyl-HSLs produced by G4, a GC-MS analysis was carried out (Fig. 4). The peaks of the products could be identified since the GC-MS properties, such as the retention times and fragmentation patterns, were identical to those of the synthetic acyl-HSLs (Table 1). Dibutyl phthalate, used as the internal standard, was extracted about 95% from the culture supernatant. As shown in Fig. 4, G4 was shown to produce C6-, C8-, C10-, and C12-HSLs, as determined by the TLC bioassays (Figs. 2 and 3). Although C6-HSL was one of the most active spots in the TLC bioassays with CV026 and NT1(pDCI41E33) (Figs. 2 and 3), it was detected at a basal level in the GC analysis, thereby demonstrating again the difference in sensitivity between bioassays and a GC analysis. The integration of the GC peak areas of C8-HSL:C10-HSL:C12-HSL showed a ratio of 3:17:1 with C10 production at 10 μM in the culture supernatant.

Involvement of Acyl-HSLs in Production of Antibiotic Substance

Quorum sensing in a clinical *B. cepacia* isolate has been shown to regulate the production of extracellular virulence factors, such as protease, lipase, and siderophores [19]. In the present study, it was found that the lagoon isolate G4 did not produce detectable protease nor lipase activities when examined as described in Materials and Methods. The possible role of the acyl-HSLs produced by G4 was investigated with autoinducer-negative mutants using Omegon-Km mutagenesis. Five strains (G4-21, -23, -25, -27, -28), partially defective in the production of autoinducers, were isolated from almost 5,000 exoconjugants. A Southern hybridization analysis showed that the Omegon-Km from pJFF350 was inserted into the G4 cellular DNA in the mutants. A double insertion of the Omegon-Km was also observed in the mutant G4-21.

The growth levels of the mutants on MSB with toluene were almost identical to that of G4, indicating that the degradation of toluene was not affected by quorum sensing in G4. In addition, the mutants were tested for their ability to produce siderophores on a CAS agar. The sizes of the orange zones surrounding the mutants and G4 were almost

Table 2. Levels of antibiotic and autoinducers produced by G4 and its mutants[a].

Strain	Antibiotic assay[b]			Autoinducer assay[c]
	24 h	48 h	72 h	
G4	1.6	1.3	–[d]	4.5
G4-21	1.1	–	–	1.0
G4-23	1.2	–	–	0.9
G4-25	1.0	–	–	1.1
G4-27	1.1	–	–	1.1
G4-28	1.0	–	–	1.0

[a]Cells were cultured in Potato dextrose broth and the values are averages from two independent experiments.
[b]Culture supernatants (each 50 µl) at a given culture time were assayed for the levels of antibiotic production using *M. luteus* KCTC1054 as the test strain, as described in Materials and Methods. The numbers are the diameters of the growth inhibition zones (cm).
[c]Culture supernatants (each 90 µl) after 48 h of culture time were assayed in petri dishes for autoinducer production, as described in Materials and Methods, using the *tra* reporter system. The numbers are the diameters of the blue zones (cm).
[d]No clear zone.

identical (ca. 0.25 cm at 4-days culture) to each other with increasing culture times. These results indicated that quorum sensing in G4 is quite different from the pathogenic *B. cepacia* isolate.

In contrast, it was found that, during the screening of the autoinducer-negative mutants, G4 showed an antibacterial activity that inhibited the growth of *A. tumefaciens*. Since this activity was weak, a more sensitive bacterium was sought to correlate the activity and quorum sensing in G4. Among our laboratory collection, *M. luteus* was the most sensitive to the substance produced by G4. The results in Table 2 show the relationship between the levels of the antibiotic substance produced by G4 and the autoinducer-negative mutants. TLC bioassay and GC-MS analysis showed that these mutants had a reduced activity to produce all acyl-HSLs, but not a specific acyl-HSL. Because the activities observed relative to the inhibition zone sizes are exponentially expressed, the difference in antibiotic activity between G4 and the mutants was about 3-fold. Therefore, it can be concluded that the mutants had a reduced activity to produce autoinducers, and also had a reduced activity to produce an antibiotic substance. Furthermore, the addition of the culture extract obtained from G4 to the culture media recovered the antibiotic activities in the mutants. This result indicates that the production of an antibiotic substance by G4 was in someway involved in the acyl-HSL-mediated quorum sensing. The production of antibiotic activity has not been previously reported from this strain. A preliminary study showed that the substance is sensitive to heat and to solvents such as ethyl acetate, and to also sensitive to protease, thereby indicating that this antibiotic substance could be a peptide or a protein.

DISCUSSION

This study demonstrated that G4 produces unsubstituted acyl-HSLs, such as C6-, C8-, C10-, and C12-HSL molecules. Other studies on pathogenic *B. cepacia* strains from patients with cystic fibrosis have indicated that at least two or three types of signaling molecules present in cell culture fluids control the production of exoproducts, such as phospholipase C, protease, and siderophores [9, 21, 35]. In addition, Lewenza *et al.* [19] showed that a different pathogenic strain, K56-2, produces C8-HSL as the predominant signaling molecule and regulates the production of virulence factors by a cell-density-dependent mechanism. In contrast, quorum sensing in G4, as shown in this study, does not modulate the production of virulence factors, but rather the biosynthesis of an antibiotic substance. The level of C8-HSL produced by K56-2 appeared to be 400-fold less than that of the C10-HSL produced by G4. The results obtained from the current study indicated that the amounts and the species of acyl-HSL molecules, and the target of autoinducer-dependent gene regulation in G4, are quite dissimilar to clinical strains. Further studies are currently ongoing to clone *luxRI* homologs from G4 to elucidate these differences in quorum sensing between *B. cepacia* strains. Many studies have been carried out to differentiate pathogenic and environmental strains [33, 36]. This was intended to eliminate the release of pathogenic *B. cepacia* into the environment for bioremediation or as a biopesticide, and also, to determinate the origins of the *B. cepacia* isolates from cystic fibrosis patients. The present study indicates that the levels and species of acyl-HSL molecules produced by *B. cepacia* could be an indicator to distinguish the pathogenic and environmental isolates as well as genomovars. Although many acyl-HSLs have been identified to produce Gram-negative strains, it would appear that this is the first report on the production of C12-HSL from bacteria. Furthermore, since strain G4 produces a series of unsubstituted acyl-HSLs in large quantities, G4 may be useful as a standard strain for identifying unsubstituted acyl-HSLs produced by bacteria.

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