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Distribution and Diversity of Acyl Homoserine Lactone Producing Bacteria from Four Different Soils

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Abstract The distribution and diversity of acyl homoserine lactone (AHL) producing bacteria in black, brown, red, and meadow soils were investigated using culture-dependent method. One out of seventy six and five out of fifty isolates from the black and the brown soil were AHL-biosensor active, respectively. They were affiliated to the genera *Ensifer* and *Pseudomonas* and the family *Enterobacteriaceae*. Thin layer chromatography showed that the most of them produced AHLs with acyl side chain of 6 or 8 carbons. No AHL-producer was found in red and meadow soils. A potential novel AHL-based quorum sensing and quenching system was identified by sequencing in the black soil isolate *Ensifer adhaerens* X097 that harbored two AHL synthetase-like proteins and one amidohydrolase-like protein. This is the first report of comparison of AHL-producers among different soils. Our data showed that composition of AHL-producers were niche specific and were not in proportion with community population.

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Introduction

Quorum sensing (QS) is a cell–cell communication system that bacteria use self-producing chemicals to coordinate their behaviors when cell density reaches certain high [6]. Several types of QS signals had been reported such as the autoinducers, small peptides, *N*-acyl homoserine lactones (AHLs), diffusible signaling factors, etc. [19]. Among these, AHLs produced by many Gram-negative bacteria were the most extensively studied ones [17]. An impressive range of bacterial activities was regulated by AHLs-based QS system such as virulence, nodulation, antibiotic production, biofilm formation, cell conjugation, growth regulation, pigmentation, etc. [16]. Laboratory study on the AHLs-based QS regulatory mechanism had made great progress during the past two decades; however, the ecological significance of AHLs-based QS remained to be explored [3].

Investigation on the distribution and diversity of AHL-producers in natural habitats provides valuable information about the ecological significance of QS in environments. Previous surveys revealed that natural niches, such as marine biofilms [10], rhizosphere and soils [2], harbored diverse AHL-producers. Using culture-dependent method, d'Angelo-Picard et al. [2] reported diverse novel AHL-producing Proteobacteria from bare soil and tobacco rhizosphere. On the other hand, culture-independent methods such as metagenomic libraries were also successfully used to screen for and identify novel LuxR/LuxI type QS system from soil [9]. These results indicated that soils were great resources for AHL-producers, and potential novel AHL-producers remain to be identified.

Bacterial compositions from bare soils were free from plant effect and represented niche specific community. Bacteria in bare soils play key roles in soil functions such as bioremediation and element cycling, etc., thus deserve

close investigation [5]. The objectives of this study were to investigate the diversity and distributions of AHL-producers from four different types of bare soils in China, and to search for bacteria with novel AHLs-based QS system.

Material and Method

Soil Sample Collection

Four distinct types of soils, the red soil, brown soil, black soil, and meadow soil, were collected from different part of China. Soil sampling locations ranged from 25°N to 43°N and from 109°E to 125°E (Table 1). All soil samples were collected in the springtime of 2011, from 20 cm depth and non-rhizosphere region. Soil samples were freshly transferred to the lab and kept in 4 °C. The pH value of soil was measured according to ISO 10390: 2005.

Bacterial Isolation

For each soil, 1 g sample was suspended in 10 ml autoclaved salty water (ASW, NaCl 0.9 %), mixed vigorously by vortex and serially diluted (10^{-1}–10^{-4}) with ASW. From each dilution, duplicate 100 µl aliquots were plated on LB agar and incubated at 28 °C for 48 h. Agar plates were then examined under a dissecting microscope (Motic, SMZ168) for colony growth, and the colony color, shape, size, surface topography, and the presence of granules were recorded. All the isolates were tested for Gram-positive or Gram-negative property by 3 % potassium hydroxide [8].

Isolation and Identification of AHL-Producers

The AHL production of each isolate from soil samples was tested by the cross-feeding bioassay using two biosensors, *Agrobacterium tumefaciens* strain A136 (pCF218) (pCF372) and *Chromobacterium violaceum* strain CV026 according to [10]. At the same time, all of the G-isolates were cultured in LB to stationary stage and the ethyl acetate (EA) extracts of their spent culture extracts were assayed for AHL activity by diffusion assay [10].

AHL-producers were identified by 16S rRNA gene [10]. Genomic DNA was extracted from isolates using a bacterial genomic DNA extraction kit (MoBio, USA). The purified PCR products were sequenced by Sangon Biotech Company (China).

Table 1 Sampling locations, pH values and numbers of total isolates, G-isolates, and AHL-producers of four soils

Soil	Sampling location	pH	No. of total isolates	No. of G-isolates	No. of AHL-producers
Black soil	125°19′E, 43°52′N	7.76	76	28	1
Meadow soil	109°51′E, 39°50′N	8.47	27	9	0
Brown soil	120°82′E, 30°15′N	7.42	50	30	5
Red soil	118°53′E, 25°22′N	5.27	23	4	0

AHL Molecular Identification by Thin Layer Chromatograph

Thin layer chromatography (TLC) was employed to identify AHL molecules. The EA extracts were loaded onto a TLC plate (20 × 20 cm TLC aluminum sheets; RP-18 F254 S) (Merck, Germany) that was subsequently developed in methanol–Millipore water (60:40 v/v). The plate was air-dried, covered with LB agar containing the biosensor *A. tumefaciens* A136, and incubated at 28 °C for 24 h. AHLs from samples were identified by comparing to standard AHLs (purchased from University of Nottingham).

Amplification of AHL Synthetase Gene from *Ensifer adhaerens* X097

Degenerate primers were designed according to conserved domains of known AHL synthetase gene sequences of *Sinorhizobium* spp. retrieved from NCBI. PCR was performed using different combination of degenerate primers to amplify the potential AHL synthetase gene from *Ensifer adhaerens* X097. PCR was carried out in 20 µl of reaction mixture containing 2 µl of X097 genomic DNA, 50 nM of degenerate primers, 1 U of *Taq* DNA polymerase (Promega), 0.25 mM of dNTPs, and 1X PCR buffer. PCR program was 1, 94 °C for 5 min; 2, 94 °C for 30 s; 3, 48 °C for 45 s; 4, 72 °C for 1 min; 5, go to 2 for 35 cycles; 6, 72 °C for 5 min; 7, 4.0 °C for ever. PCR products were cloned and sequenced using pMD19 T vector (TaKaRa, CHN) according to the manufacture instruction. To get longer sequence, genome walking was performed by arbitrary PCR using new primers designed based on the obtained sequence and arbitrary primers (Table 3) [11]. Sequence was analyzed by BLASTX and ORF finder in NCBI.

Accession Numbers

Partial sequences of 16S rRNA gene of these AHL-producers were submitted to the GenBank under accession numbers JQ390527–JQ390532. Two genomic DNA fragments containing AHL synthetase genes and amidohydrolase gene from *Ensifer adhaerens* X097 were submitted to the GenBank under accession numbers JX239991–JX239992.

Result

Distribution of AHL-Producers in Each Soil

The red soil, brown soil, black soil, and meadow soil were distinguishable from their appearance (Fig. 1). The pH values of four soils ranged from acid to alkaline. The red soil was an acid soil with pH value of 5.27. The brown and black soils were slightly alkaline soils with pH values of 7.42 and 7.76, respectively. The meadow soil was an alkaline soil with pH value of 8.47 (Table 1).

Isolation record showed that the black soil had the highest bacterial diversity with 76 isolates in total and 28 of them (36.8 %) were Gram negative. For the brown soil, 50 isolates were obtained and 30 of them (60 %) were Gram negative (Table 1). The meadow soil and red soil contained much less diverse bacteria with 27 and 23 total isolates, 9 and 4 Gram-negative isolates, respectively.

Fig. 1 Samples of four bare soils

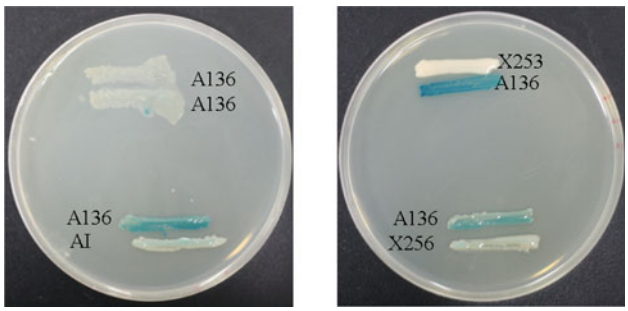

Fig. 2 Representative plates of cross-feeding assay for screening of AHL-producers using biosensor A136. Control plate includes A136 versus A136 as negative control and AI (known AHL-producer) versus A136 as positive control

Screening for AHL-producers resulted in five strains from the brown soil (10 %) and one from the black soil (Table 1). Representative screening plates were shown in Fig. 2. No AHL-producer was found from red and meadow soils.

Diversity of AHL-Producers

Sequence analysis of 16S rRNA genes showed that all six AHL-producers belong to Proteobacteria. Among them, four were gamma-proteobacteria, affiliated to the genus *Pseudomonas* or the family *Enterobacteriaceae*; the other two belonged to the alpha-proteobacteria and affiliated to the genus *Ensifer* (Table 2). Isolates X097/X253, X212, and X256 were closely related to *Ensifer adhaerens*, *Enterobacter ludwigii*, and *P. migulae* that had never been reported as AHL-producers before. Thus, these isolates extended the list of AHL-producing species.

AHL Profiles Identified by TLC

The AHL profiles from these AHL-producers were analyzed by TLC (Fig. 3). AHL molecular diversity was summarized in Table 2. The two *Enterobacteriaceae* produced only one chemical that triggered the A136 coloration. The *Enterobacter ludwigii* X212 produced C6-HSL, whereas the molecule from *Hafnia alvei* X229 could not be identified yet. The *P. putida* X236 produced C6-HSL, while the *P. migulae* X256 produced 3-OH–C8–HSL and another compound that remained to be identified. The two strains closely related to *Ensifer* both produced multiple AHL molecules including 3-oxo-C6-HSL and 3-oxo-C8-HSL, and some others remained to be identified.

AHL Synthetase Gene from *Ensifer adhaerens* X097

Among several pairs of degenerate primers designed, SRAHL352F and SRAHL436R (Table 3) successfully amplified around 100 bp PCR products. Clone sequencing showed that the 100 bp PCR products contained two

Table 2 Phylogenetic identification and AHLs profiles of AHL-producers from brown and black soils

Soil	Isolate	Closest match in the GenBank (similarity)	AHLs profile
Brown soil	X212	*Enterobacter ludwigii* (99 %)	C6–HSL
	X229	*Hafnia aivei* (99 %)	NI
	X236	*Pseudomonas putida* (100 %)	C6–HSL
	X256	*Pseudomonas migulae* (99 %)	3-OH–C8; NI
	X253	*Ensifer adhaerens 4CCS7* (100 %)	3-OXO-C6; 3-OXO-C8; NI, NI
Black soil	X097	*Ensifer adhaerens 33499* (100 %)	3-OXO-C6; 3-OXO-C8; NI

NI not identified (spots on TLC plates did not correspond to any known standard)

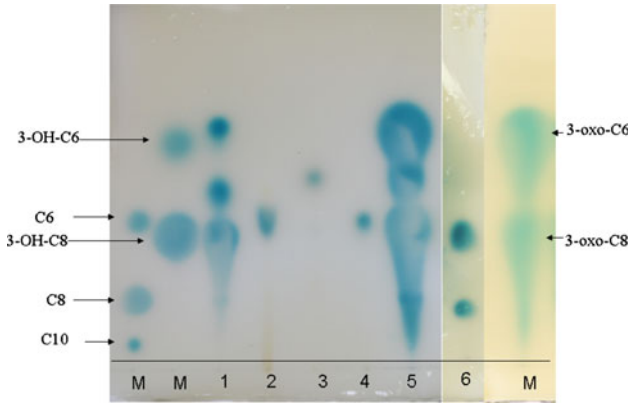

Fig. 3 TLC analysis of AHL profiles from six AHL-producers. The presence of AHLs was visualized by biosensor A136. *1* X097 *Ensifer adhaerens* 33499, *2* X212 *Enterobacter ludwigii*, *3* X229 *Hafnia alvei*, *4* X236 *P. putida*, *5* X253 *Ensifer adhaerens* 4CCS7, *6* X256 *P. migulae*. *M* standard AHLs

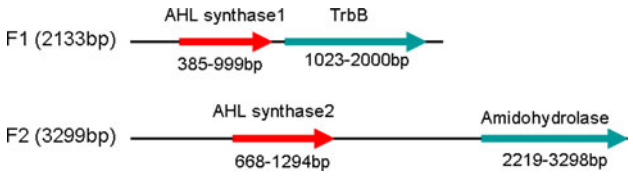

Fig. 4 Gene arrangements on the two genomic DNA fragments F1 and F2 from X097 *Ensifer adhaerens*. The AHL synthetase genes are highlighted in *red*, and the other genes are highlighted in *blue* (Color figure online)

different fragments; BLASTX analysis indicated that both were closely related to AHL synthetase gene. Genome walking successfully extended the two fragments to 2,133 bp (fragment 1) and 3,299 bp (fragment 2) (Fig. 4). BLASTX and ORF finder analysis showed that fragment 1 contained a gene (615 bp) that was 74 % similar to the AHL synthetase gene from *Sinorhizobium fredii*. Closely down stream, a gene was found with 88 % similarity to the TrbB of *Sinorhizobium meliloti*. The fragment 2 contained another gene (627 bp) that was 83 % similar to AHL synthetase gene of *Sinorhizobium meliloti* SM11. Down stream, a partial gene (1,080 bp) was found with 57 % similarity to amidohydrolase of *A. tumefaciens*. These two AHL synthetase genes shared 58.2 % similarity. These results indicated that *Ensifer adhaerens* X097 harbored two novel AHL synthetase genes and at the same time carried a novel gene for AHLs degrading enzyme—amidohydrolase.

Discussion

Many factors affect the composition and abundant of bacterial diversity in a soil, such as organic matters, pH value, moisture, etc. Although not measured in this study, generally, organic matter contents in the four soils decreased in the order of black soil, brown soil, red soil, and meadow soil [7]. In this study, the black soil contained the most diverse total cultivable bacterial species, probably due to the high organic matter level. However, the brown soil had the highest diversity of Gram-negative bacteria, and consequently, the most diverse AHL-producers. AHL-producers made up of 10 % diversity in brown soil, which is close to average 8 % that was previously reported [2]. On the other hand, it is known that AHL molecules are sensitive to pH value [17]. The low pH value in red soil and the high pH value in meadow soil may be one of negative selection factors for AHL-producing species, as no AHL-producers were found in these two soils.

All of the six AHL-producers isolated in this study belonged to known AHL-producing genus, except that the *Ensifer* could be regarded as the novel AHL-producing group. The group *Ensifer* is closely related to the group *Sinorhizobium*; they were proposed to merge under the genus name *Ensifer* [18]. Although AHL-producers in *Sinorhizobium* spp. had been reported, AHL-producing *Ensifer* had never been reported. *Ensifer adhaerens* was regarded as the type species within its genus, and was originally reported as a bacterial predator in soil [1]. This is the first report of AHL production in *Ensifer adhaerens.* It would be very interesting to further study whether the predatory behavior is regulated by QS or not.

Degenerate primers designed from the conserved domains of AHL synthetase in *Sinorhizobium* spp. successfully amplified homologs from the *Ensifer adhaerens* X097. It was reported that there were two QS systems in *Sinorhizobium meliloti* and strains of this species produced multiple AHL molecules [15]. Similarly, two novel AHL synthetase genes and multiple AHLs were identified from X097. The two AHL synthetase genes shared 58.2 %

Table 3 Primers used in this study

Primer	Sequence (5′–3′)	Source
SRAHL352F	TCGATGAT(C/G)CC(C/G)GC(A/G)AACAT	This study
SRAHL436R	ATG(G/A)TCGA(A/G)AGCTC(C/G)CGCTT	This study
ARB1	GGCCACGCGTCGACTAGTACNNNNNNNNN NGATAT	Huang et al. [11]
ARB2	GGCCACGCGTCGACTAGTAC	Huang et al. [11]

similarity, and may correspond for synthesis of different sets of AHL molecules, which deserves further investigation. More interesting, in one fragment we found an amidohydrolase-like gene down stream to the AHL synthetase gene. Amidohydrolase is the enzyme that can degrade AHL molecules [4]. These results indicated that X097 carried a quorum quenching system as well. Recently, involvement of multi-loci in quorum quenching of autoinducer I molecules in a nitrogen-fixing symbiont *Rhizobium* (*Sinorhizobium*) sp. NGR234 was also reported [14].

The bacterial family *Enterobacteriaceae* contains more than 40 genera and quite a few of them are clinical relevant, including *Enterobacter* and *Hafnia* [12]. The resolution of 16S rRNA for this group was limited and intrafamily DNA relatedness values were high [12]. The utility of 16S rDNA sequence to study the family *Enterobacteriaceae* should be carefully interpreted. In this study, isolates X212 and X229 showed 99 % similarity to *Enterobacter* and *Hafnia.* Both of them should be further characterized in order to confirm their phylogenetic affiliation. Moreover, identification of AHL molecule in X229 remained uncertain. Nevertheless, isolate X212 extended the AHL-producing species in the family *Enterobacteriaceae*.

Some of the model strains for QS studies were from the genus *Pseudomonas*, such as *P. aeruginosa* and *P. fluorescens.* However, the distribution of AHL-producers on species level in this genus has not been fully surveyed yet [13]. In this study, we obtained two *Pseudomonas* spp. that produced AHLs. Isolate X256 was 99 % similar to *P. migulae* that had never been reported to produce AHL, thus extending the AHL-producer list in Pseudomonades.

The isolation of AHL-producers in this study was based on culture-dependent method. Because some AHLs are synthesized only under particular environmental conditions, our approach is likely to under-represent the occurrence of AHL-producers. The success in amplifying AHL synthetase gene in this study suggested that at genus level, PCR method with degenerate primers could be a great alternative method. It was reported that PCR methods using QS genes was successfully established to detect *Serratia* spp. [20]. In the future, the primer set would be useful in conducting culture-independent investigation of AHL-producing *Ensifer*.

Many soil functions are related with bacterial activities. It is possible to achieve soil function through controlling soil bacterial activity via QS given the strong regulatory nature of QS [3, 16]. Investigation on distribution and diversity of AHL-producers greatly increased our knowledge about the potential of cell–cell communication in natural niches, and provided valuable information for in situ control of bacterial activities.

In conclusion, this study reported for the first time the distribution and diversity of AHL-producers in four different soils. Our data showed that compositions of AHL-producers were soil niche specific and were not in proportion with community population. The potential sophisticated AHL-based QS and quorum quenching system in *Ensifer adhaerens* X097 was also revealed for the first time.

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References

1. Casida LE (1982) *Ensifer Adhaerens* gen. nov., sp. nov.: a bacterial predator of bacteria in soil. Int J Syst Bacteriol 32:339–345
2. d'Angelo-Picard C, Faure D, Penot I, Dessaux Y (2005) Diversity of *N*-acyl homoserine lactone-producing and -degrading bacteria in soil and tobacco rhizosphere. Environ Microbiol 7:1796–1808
3. Decho A, Norman R, Visscher P (2010) Quorum sensing in natural environments: emerging views from microbial mats. Trends Microbiol 18:73–80
4. Dessaux Y, Chapelle E, Faure D (2011) Quorum sensing and quorum quenching in soil ecosystems. In: Witzany G (ed) Biocommunication in soil microorganisms: soil biology, vol 23. Springer, Berlin, pp 339–367
5. Eilers KG, Debenport S, Anderson S, Fierer N (2012) Digging deeper to find unique microbial communities: the strong effect of depth on the structure of bacterial and archaeal communities in soil. Soil Biol Biochem 50:58–65
6. Fuqua C, Winans SC, Greenberg EP (1996) Census and consensus in bacterial ecosystems: the LuxR–LuxI family of quorum-sensing transcriptional regulators. Ann Rev Microbiol 50:727–751
7. Gen ZC, Dai W (2011) Agrology. Science publishing, Shanghai
8. Gregersen T (1978) Rapid method for distinction of Gram-negative from Gram-positive bacteria. Eur J Appl Microbiol Biotechnol 5:123–127
9. Hao Y, Winans SC, Glick BR, Charles TC (2010) Identification and characterization of new LuxR/LuxI-type quorum sensing systems from metagenomic libraries. Environ Microbiol 12:105–117
10. Huang YL, Ki JS, Case R, Qian PY (2008) Diversity and acyl-homoserine lactone production among subtidal biofilm forming bacteria. Aquat Microb Ecol 52:185–193
11. Huang YL, Li M, Yu ZL, Qian PY (2011) Correlation between pigmentation and larval settlement deterrence by *Pseudoalteromonas* sp. sf57. Biofouling 27:287–293
12. Janda JM, Abbott SL (2006) The genus *Hafnia*: from soup to nuts. Clin Microbiol Rev 19:12–28
13. Juhas M, Eberl L, Tummler B (2005) Quorum sensing: the power of cooperation in the world of *Pseudomonas*. Environ Microbiol 7:459–471
14. Krysciak D, Schmeisser C, Preuß S, Riethausen J, Quitschau M, Grond S, Streit WR (2011) Involvement of multiple loci in quorum quenching of autoinducer I molecules in the nitrogen-fixing symbiont *Rhizobium* (*Sinorhizobium*) sp. strain NGR234. Appl Environ Microbiol 77:5089–5099
15. Marketon MM, Gonzalez JE (2002) Identification of two quorum-sensing systems in *Sinorhizobium meliloti*. J Bacteriol 184:3466–3475
16. von Bodman SB, Willey JM, Diggle SP (2008) Cell–cell communication in bacteria: united we stand. J Bacteriol 190:4377–4391

17. Williams P (2007) Quorum sensing, communication and cross-kingdom signalling in the bacterial world. Microbiol-SGM 153: 3923–3938
18. Young JM (2003) The genus name *Ensifer* Casida 1982 takes priority over *Sinorhizobium* Chen et al. 1988, and *Sinorhizobium morelense* Wang et al. 2002 is a later synonym of *Ensifer adhaerens* Casida 1982. Is the combination '*Sinorhizobium adhaerens*' (Casida 1982) Willems et al. 2003 legitimate? Request for an opinion. Int J Syst Evol Microbiol 53:2107–2110
19. Zhang LH, Dong YH (2004) Quorum sensing and signal interference: diverse implications. Mol Microbiol 53:1563–1571
20. Zhu H, Sun SJ, Dang HY (2008) PCR detection of *Serratia* spp. using primers targeting pfs and luxS genes involved in AI-2-dependent quorum sensing. Curr Microbiol 57:326–330