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Isolation and identification of quorum quenching bacteria from environmental samples

Steven E.A. Christiaen, Gilles Brackman, Hans J. Nelis, Tom Coenye *

Laboratory of Pharmaceutical Microbiology, Ghent University, Harelbekestraat 72, B-9000 Ghent, Belgium

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ABSTRACT

A large number of Gram-negative pathogens produce N-acylhomoserine lactones (AHLs) as signal molecules for quorum sensing (QS). This cell–cell communication system allows them to coordinate gene expression and regulate virulence. Therefore, strategies to inhibit QS are promising for the control of infectious diseases. The aim of the present study was to develop a high-throughput method for the isolation and identification of AHL-degrading bacteria from environmental samples. Samples were cultured in a microtitre plate in a minimal medium containing 1 mM N-(3-oxo-dodecanoyl)-L-homoserine lactone and 2 mM N-(3-oxo-hexanoyl)-L-homoserine lactone as the sole sources of carbon and nitrogen. Isolates growing on this minimal medium were subcultured and identified by partial 16S rRNA gene sequencing. Subsequently, the AHL-degrading capacity of each isolate was evaluated in the *Pseudomonas aeruginosa* QSIS2 biosensor assay, as such or after treatment with heat or proteinase K. The 16 samples tested yielded a total of 59 isolates which are, either alone or as part of a consortium, able to use AHL signal molecules as sole sources of carbon and nitrogen. Follow-up experiments have shown that in each sample there is at least one isolate with quorum quenching (QQ) activity, and that for all samples combined, 41 isolates have QQ activity. Furthermore, heat treatment did not fully inhibit QQ activity in all isolates. In some isolates, QQ activity was lost after proteinase K treatment, while others remained able to quench QS. Therefore, it is likely that some isolates produce and secrete (a) heat-stable, low molecular weight inhibitory compound(s).

1. Introduction

A large number of Gram-negative bacteria produce N-acylhomoserine lactones (AHLs) as signal molecules for quorum sensing (QS) (Winzer and Williams, 2001). AHLs are diffusible, low molecular weight molecules that are characterized by a conserved lactone moiety and a variable acyl side chain. Signal molecules from different species typically differ in side chain length, saturation and substitution at the third carbon atom. AHLs are produced by a LuxI homologue and are subsequently released into the extracellular environment, resulting in the accumulation of the signal molecule. Once a certain threshold concentration has been reached, the AHLs bind to a cytoplasmatic LuxR homologue, serving as a transcriptional activator. Upon ligand binding, the LuxR-AHL complexes bind to promoter regions of target genes, resulting in the activation or repression of the expression of specific genes (De Kievit and Iglewski, 2000; Camilli and Bassler, 2006).

This type of cell–cell communication system allows bacteria to coordinate gene expression and regulate the production of virulence factors in a cell-density dependent way (Fuqua et al., 1994; De Kievit and Iglewski, 2000; Fuqua and Greenberg, 2002). Genes encoding these virulence factors controlled by QS regulation include, but are not limited to plasmid conjugation in *Agrobacterium tumefaciens*, biofilm formation and siderophore production in *Burkholderia cenocepacia*, exoprotease and exotoxin production in *Pseudomonas aeruginosa* and swarming motility in *Serratia* sp. (Coenye, 2010; Williams et al., 2007; Von Bodman et al., 2003). Therefore, QS is a potential therapeutic target, and the quenching of bacterial cell–cell communication could be a promising strategy to attenuate virulence and thus control infections. Furthermore, interfering with QS rather than using bactericidal or bacteriostatic drugs to combat bacterial infections is a highly attractive concept since it is less likely to induce the development of bacterial resistance (Cámara et al., 2002; Costerton et al., 2007; Hentzer and Givskov, 2003).

Besides the use of a wide range of small molecule inhibitors of QS (Brackman et al., 2009; Galloway et al., 2011), the enzymatic degradation of AHLs has been described as a possible approach to interfere with QS (Dong et al., 2000; Dong et al., 2001; Oger and Uroz, 2010; Stoltz et al., 2008). Thus far, two major types of AHL-degrading enzymes have been extensively studied: AHL acylases (or AHL amidases) and AHL lactonases. Members of the former family cleave the molecule into a free homoserine lactone and a fatty acid, while members of the latter family hydrolyze the lactone ring, yielding a

* Corresponding author. Tel.: +32 9 264 81 41; fax: +32 9 264 81 95.
E-mail addresses: Steven.Christiaen@UGent.be (S.E.A. Christiaen), Gilles.Brackman@UGent.be (G. Brackman), Hans.Nelis@UGent.be (H.J. Nelis), Tom.Coenye@UGent.Be (T. Coenye).

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Table 1
Samples taken from various locations with their specifications.

Sample code	Location	Source
P	Desk plant	Rhizosphere (*Cactaceae*)
Li1	Greenhouse (Ghent, Belgium)	Rhizosphere (*Araceae*)
Li2	Greenhouse (Ghent, Belgium)	Rhizosphere (*Rutaceae*)
Li3	Greenhouse (Ghent, Belgium)	Rhizosphere (*Asteraceae*)
Li4	Greenhouse (Ghent, Belgium)	Rhizosphere (*Caricaceae*)
MP1	City Park (Ghent, Belgium)	Rhizosphere (*Fagaceae*)
MP2	City Park (Ghent, Belgium)	Rhizosphere (*Gramineae*)
MP3	City Park (Ghent, Belgium)	Pond water
Le1	Botanical Garden (Ghent, Belgium)	Rhizosphere (*Plumbaginaceae*)
Le2	Botanical Garden (Ghent, Belgium)	Rhizosphere (*Brassicaceae*)
Le3	Botanical Garden (Ghent, Belgium)	Rhizosphere (*Aristolochiaceae*)
Le4	Botanical Garden (Ghent, Belgium)	Rhizosphere (*Geraniaceae*)
Le5	Botanical Garden (Ghent, Belgium)	Rhizosphere (*Fabaceae*)
TC	Aquarium	Water
Lw1	Botanical Garden (Ghent, Belgium)	Pond water (outdoor)
Lw2	Botanical Garden (Ghent, Belgium)	Pond water (indoor)

homoserine. Degradation products of both enzymes are no longer active, thus shutting down the QS circuitry (Amara et al., 2011; Czajkowski and Jafra, 2009). Finally, AHL oxidoreductase, a third type of AHL inactivating enzyme, has also been described (Uroz et al., 2005).

The procedures described to isolate quorum quenching (QQ) bacteria mostly involve cultivation of isolates on general media and subsequent tests to check whether or not the isolates possess AHL-degrading activity (Jafra et al., 2006). This implies that a lot of time, effort and costs are put in testing non-QQ bacteria, since not all environmental isolates are capable of degrading AHLs. Another approach is the cultivation of samples in a minimal medium supplemented with a single AHL as the sole source of carbon and nitrogen (Chan et al., 2009). Although this method already narrows down the number of isolates to be screened, it is likely that a large number of QQ positive strains will go undetected because AHL degradation specificity is dependent on side chain length. In the present study we report the development of a novel approach for the isolation and identification of QQ bacteria, and its validation based on the isolation of QQ bacteria from 16 environmental samples.

2. Materials and methods

2.1. Sample preparation and isolation of QQ consortia from environmental samples

Locations from which samples were taken are listed in Table 1. 1 g of sample material (soil or water) was suspended in 10 ml of saline (0.9% NaCl) and subjected to three cycles of 30 s vigorous vortexing and 30 s sonication. After briefly spinning down the sample at 3000 rpm to remove larger particles and debris, 2 ml of the resulting supernatant was centrifuged for 4 min at 4500 rpm. Subsequently, pellets were resuspended in 5 ml saline and 250 µl of this inoculum suspension was added to the wells of a 24-well microtitre plate (MTP). Subsequently, 200 µl of a minimal medium was added, together with 50 µl of a mixture of AHLs (final concentrations: 2 mM N-(3-oxo-hexanoyl)-L-homoserine lactone (3OC6HSL) and 1 mM N-(3-oxo-dodecanoyl)-L-homoserine lactone (3OC12HSL); Sigma Aldrich, Bornem, Belgium). The minimal isolation medium (MIM), based upon a previously described medium (Chan et al., 2009) contained (per litre) NaCl, 1 g; KCl, 0.5 g; $MgCl_2$, 0.4 g; $CaCl_2$, 0.1 g; Na_2SO_4, 0.15 g; KH_2PO_4, 2 g; Na_2HPO_4, 2.25 g. Subsequently, the medium was acidified to pH 5.5 with 1 M HCl, and trace elements were added to final concentrations of 1 mg $FeCl_3$, 0.1 g $MnCl_2$ and 46 mg $ZnCl_2$ per litre. Finally, the medium was filter sterilized through a 0.22 µm filter. After incubation for 3 days at room temperature, 100 µl of the cultures was transferred to 1 ml fresh medium for enrichment. This was repeated and after the third enrichment cycle, an aliquot of 100 µl was taken from the samples and streaked on tryptone soy agar (TSA, Lab M, Bury, United Kingdom). After incubation for 48 h at room temperature, colonies with different morphological appearance were separated, subcultured on TSA and stored at −80 °C in Microbank vials (Pro-Lab Diagnostics, Richmond Hill, Canada).

2.2. Detection of QQ in bacterial consortia

The QS inhibition selector (QSIS) strain *P. aeruginosa* QSIS2 was cultured aerobically at 37 °C in ABT minimal medium (AB medium, containing 2.5 mg/l of thiamine), supplemented with 0.5% glucose, 0.5% casamino acids and 80 µg/ml gentamicin (Rasmussen et al., 2005). This reporter strain is a *P. aeruginosa lasI rhlI* double mutant harboring pLasB-SacB1 encoding an AHL-induced killing system. A QSIS2 assay was developed in MTP format based on the assay described by Rasmussen et

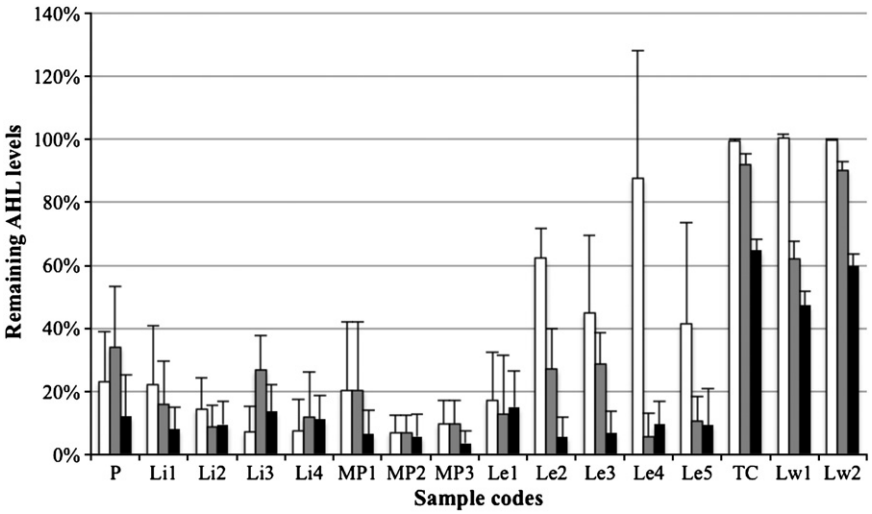

Fig. 1. Remaining AHL levels for all samples in the diluted sterile supernatant of the microbial consortia cultures after the first (white bars), second (grey bars), and third (black bars) enrichment cycle, compared to a non-inoculated control ($n \geq 12$, means ± standard deviation).

al. (2005) and was performed as described previously (Brackman et al., 2009). To detect AHL degradation in the minimal medium after each enrichment cycle, the spent medium was filter sterilized and diluted (1:100). Subsequently, 50 µl of the sterile supernatant was added to the well of a MTP, containing the QSIS2 biosensor (total volume 200 µl). Non-inoculated MIM with AHLs served as a positive control.

2.3. Detection of QQ activity in bacterial isolates

To detect total QQ activity of the isolates, 900 µl of 24 h cultures in tryptone soy broth (TSB, Lab M, Bury, United Kingdom), obtained after incubation at 37 °C, was mixed with a mixture of N-butyryl-DL-homoserine lactone (C4HSL) and 3OC12HSL (final concentration of 800 nM for each AHL in the reaction mixture). After 24 h incubation at 30 °C, the reaction mixtures were filter sterilized and 50 µl was added to the wells of a MTP containing the QSIS2 biosensor, as described above. Non-inoculated TSB mixed with sterile MilliQ water or AHLs served as negative controls (0% activation of the QS system) and positive controls (100% activation of the QS system), respectively. To detect the presence of extracellular QQ activity, 24 h old cultures (TSB, 37 °C) were filter sterilized before being mixed with the AHLs.

Table 2
Results of the partial 16S rRNA gene sequencing experiments and remaining AHL levels as measured in the QSIS2 assay ($n \geq 12$). Data shown are the average (±SEM) of the remaining AHL levels in the sterile supernatant of the isolates after incubation of the AHL mixture with isolate cultures. Values that are significantly lower ($p \leq 0.05$) than these from positive controls are marked with an asterisk. Isolates for which no remaining AHL levels could be detected are marked with ND (could not be detected).

Sample	Isolate code	Identity of closest match	Accession no.	Remaining AHL levels	
P	P1	*Leifsonia* sp.	JN014009	106 ± 5%	
	P2	*Kocuria rhizophila*	JN014010	101 ± 5%	
	P3	*Microbacterium shrimpcida*	JN014011	28 ± 5%	*
Li1	Li1-1	*Arthrobacter* sp.	JN014012	37 ± 3%	*
	Li1-2	*Comamonas* sp.	JN014013	90 ± 4%	*
	Li1-3	*Advenella incenata*	JN014014	103 ± 4%	
Li2	Li2-1	*Arthrobacter nicotinovorans*	JN014015	70 ± 5%	*
	Li2-2	*Comamonas testosteroni*	JN014016	4 ± 3%	*
	Li2-3	*Agrobacterium tumefaciens*	JN014017	104 ± 5%	
Li3	Li3-1	*Arthrobacter nicotinovorans*	JN014018	ND	*
	Li3-2	*Pseudomonas putida*	JN014019	6 ± 6%	*
	Li3-3	*Sinorhizobium* sp.	JN014020	120 ± 8%	
Li4	Li4-1	*Ochrobactrum anthropi*	JN014021	107 ± 4%	
	Li4-2	*Pseudomonas* sp.	JN014022	ND	*
	Li4-3	*Arthrobacter* sp.	JN014023	5 ± 4%	*
MP1	MP1-1	*Pseudomonas putida*	JN014024	58 ± 7%	*
	MP1-2	*Arthrobacter* sp.	JN014025	ND	*
	MP1-3	*Stenotrophomonas rhizophila*	JN014026	76 ± 11%	*
	MP1-4	*Stenotrophomonas rhizophila*	JN014027	103 ± 2%	
MP2	MP2-1	*Pseudomonas putida*	JN014028	ND	*
	MP2-2	*Pseudomonas* sp.	JN014029	89 ± 4%	*
	MP2-3	*Stenotrophomonas rhizophila*	JN014030	32 ± 8%	*
	MP2-4	*Delftia* sp.	JN014031	15 ± 4%	*
	MP2-5	*Achromobacter xylosoxidans*	JN014032	72 ± 4%	*
	MP2-6	*Alcaligenes* sp.	JN014033	44 ± 8%	*
MP3	MP3-1	*Pseudomonas* sp.	JN014034	52 ± 6%	*
Le1	Le1-1	*Staphylococcus* sp.	JN014035	88 ± 6%	*
	Le1-2	*Delftia* sp.	JN014036	2 ± 5%	*
	Le1-3	*Arthrobacter nicotinovorans*	JN014037	4 ± 8%	*
	Le1-4	*Arthrobacter aurescens*	JN014038	46 ± 6%	*
Le2	Le2-1	*Arthrobacter* sp.	JN014039	4 ± 6%	*
	Le2-2	*Achromobacter xylosoxidans*	JN014040	97 ± 4%	
	Le2-4	*Delftia* sp.	JN014041	13 ± 6%	*
	Le2-5	*Delftia* sp.	JN014042	ND	*
	Le2-6	*Kocuria* sp.	JN014043	112 ± 9%	
Le3	Le3-1	*Stenotrophomonas rhizophila*	JN014044	65 ± 18%	*
	Le3-2	*Aeromonas hydrophila*	JN014045	116 ± 16%	
	Le3-3	*Arthrobacter* sp.	JN014046	2 ± 7%	*
	Le3-4	*Pseudomonas* sp.	JN014047	9 ± 9%	*
	Le3-5	*Pseudomonas* sp.	JN014048	15 ± 6%	*
Le4	Le4-1	*Achromobacter denitrificans*	JN014049	102 ± 3%	
	Le4-2	*Achromobacter denitrificans*	JN014050	4 ± 7%	*
	Le4-3	*Pseudomonas* sp.	JN014051	ND	*
	Le4-4	*Pseudomonas alcaligens*	JN014052	5 ± 6%	*
	Le4-5	*Pseudomonas putida*	JN014053	ND	*
Le5	Le5-1	*Paracoccus yeei*	JN014054	106 ± 5%	
	Le5-2	*Arthrobacter nicotinovorans*	JN014055	4 ± 6%	*
	Le5-3	*Brevundimonas* sp.	JN014056	8 ± 4%	*
	Le5-4	*Brevundimonas bullata*	JN014057	0 ± 3%	*
TC	TC1	*Agrobacterium tumefaciens*	JN014058	114 ± 4%	
	TC2	*Aeromonas* sp.	JN014059	120 ± 5%	
	TC3	*Shewanella xiamenensis*	JN014060	24 ± 4%	*
Lw1	Lw1-1	*Diaphorobacter* sp.	JN014061	27 ± 5%	*
	Lw1-2	*Aeromonas media*	JN014062	127 ± 4%	
	Lw1-3	*Aeromonas hydrophila*	JN014063	116 ± 4%	
Lw2	Lw2-1	*Aeromonas media*	JN014064	127 ± 4%	
	Lw2-2	*Delftia* sp.	JN014065	47 ± 3%	*
	Lw2-3	*Citrobacter* sp.	JN014066	128 ± 4%	
	Lw2-4	*Aeromonas* sp.	JN014067	132 ± 4%	

2.4. Elucidation of the mechanism of the QQ activity

To determine whether the QQ activity is due to enzymatic degradation of the AHL molecules, samples of QQ positive isolates were heat-treated or treated with proteinase K prior to the QSIS2 assay. For heat treatment, samples were autoclaved (121 °C, 20 min) before addition to the AHL mixture. For the proteinase K treatment, 850 µl of a 24 h old culture in TSB (37 °C) was mixed with 100 µl of a proteinase K solution (500 µg/ml) (Sigma Aldrich, Bornem, Belgium) and incubated for 24 h at 37 °C, before supplementation with AHLs. To further investigate the mechanisms of QQ activity, the supernatant of Le1-2 and Lw1-1 (two of the most active isolates) with extracellular QQ activity) was fractionated by size exclusion chromatography (SEC). To this end gravity flow columns with a cut-off value of 6000 Da (Econo-Pac 10DG) were used according to the minimal dilution protocol instructions supplied by the manufacturer (Bio-Rad Laboratories SA/NV, Eke, Belgium). In brief, 3 ml of sterile supernatant was poured onto the column and a first fraction, containing the higher molecular weight components (>6000 Da), was eluted with 4 ml MilliQ water. Subsequently, smaller molecular weight components were eluted with 8 ml of MilliQ water, yielding 4 subsequent fractions of 2 ml each. The fractions obtained were then again tested for QQ activity in the same way as described above.

2.5. Identification of the isolates

Total DNA of the isolates was obtained by alkaline lysis. Isolates were tentatively identified by partial 16S rRNA gene sequencing (Big Dye Terminator, Applied Biosystems, Halle, Belgium) using pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pD (5′-GTA TTA CCG CGG CTG CTG-3′) as forward and reverse primers, respectively (Coenye et al., 1999). Sequencing was performed using a 96-capillary 3730*xl* DNA Analyzer (Applied Biosystems). Sequence data were processed with the BioEdit software application to align and make consensus sequences. The closest related sequences were obtained using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.6. Statistical analysis

Statistical analysis was performed using SPSS 16.0 software. The non-parametric Mann-Whitney test was used to compare the results.

3. Results and discussion

3.1. Detection of AHL degradation by bacterial consortia from environmental samples

In Fig. 1 the remaining AHL levels for each sample in the diluted sterile supernatant from the microbial consortia after the first, second, and third enrichment cycles are shown. Compared to a non-inoculated control (100% QS), all samples show a decrease in AHL levels in the spent medium after culture and enrichment. As would be expected, for most samples AHL levels decrease further with every enrichment cycle. Furthermore, these results indicate the presence of bacteria able to use AHLs as sole sources of C and N in every sample investigated. However, these data do not allow us to differentiate between isolates that are able to degrade AHLs on their own, or those that can use degradation products of AHLs produced by other members of the consortium.

3.2. Identification of the isolates

The results of the partial 16S rRNA gene sequencing experiments are shown in Table 2. The 59 isolates were classified in 21 genera. 75% (44) of the isolates were Gram-negative. Most frequently encountered genera are *Pseudomonas* (11 isolates), *Arthrobacter* (10), *Aeromonas* (6), *Delftia* (5) and *Stenotrophomonas* and *Achromobacter* (4 isolates each).

3.3. Detection of QQ activity in bacterial isolates

The results of the experiments on the QQ activity of the individual isolates are shown in Table 2. Additionally, the extracellular QQ activity of isolates for which the remaining AHL levels are significantly ($p \leq 0.05$) lower than the positive control (100% QS) is shown in Fig. 2.

Adding isolates cultured in TSB to an AHL mixture leads to quenching of QS for 41 of the 59 isolates. Furthermore, our results indicate that among the taxa recovered, *Pseudomonas* spp., *Arthrobacter* spp., and *Delftia* spp. showed the highest QQ activity. Remarkably, most genera to which QQ isolates obtained in the present study belong, have previously been described in the literature for their ability to degrade AHLs, supporting the hypothesis that our method is indeed a valid high-throughput alternative for time-consuming and more expensive protocols (Wang et al., 2010; Yang et al., 2006; Uroz et al., 2007; Jafra et al., 2006).

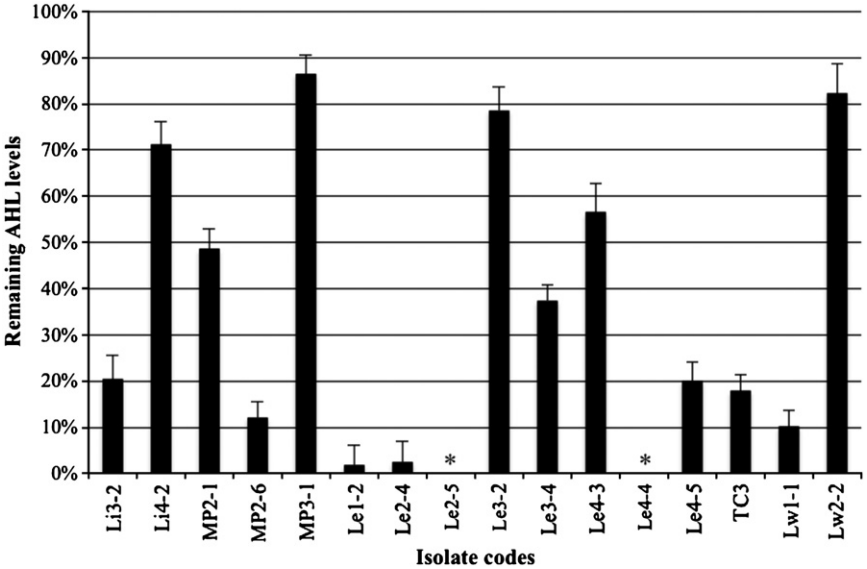

Fig. 2. Extracellular QQ activities of the isolates. Data shown are the averages (±SEM) of the remaining AHL levels in the sterile supernatant of the isolates after incubation of the AHL mixture with sterile culture supernatant ($n \geq 12$). Isolates for which no remaining AHL levels could be detected are indicated by an asterisk replacing the bar.

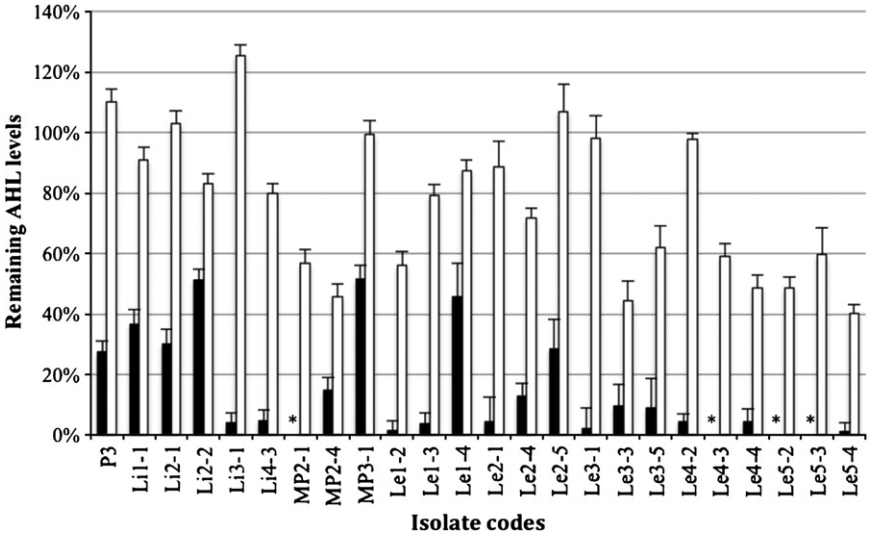

Fig. 3. Remaining AHL levels after heat treatment. Data shown are the remaining AHL levels (averages ± SEM) in the sterile supernatant of the isolates after incubation of the AHLs with untreated (black bars) and heat-treated cell cultures (white bars) ($n \geq 12$). Isolates for which no remaining AHL levels could be detected are indicated by an asterisk replacing the bar.

We also tested the co-incubation of the cell-free supernatant with an AHL mixture. A limited number of isolates (14) produced and secreted molecules interfering with QS. The majority of the isolates with extracellular QQ activity belonged to the genera *Pseudomonas* and *Delftia* (7 and 3 isolates, respectively).

3.4. Remaining QQ activity after heat treatment

In Fig. 3 the remaining AHL levels in the heat-treated culture supernatant are shown for isolates yielding significantly ($p \leq 0.05$) higher values for heat-treated samples than untreated ones. Some isolates retained QQ activity, suggesting the production of a heat-stable QQ molecule. The existence of thermal resistance and thermophilicity within the family of microbial lactonases in thermophilic archaea has recently been shown by Mandrich et al. (2010). Our findings suggest that other microbial lactonases might also be thermostable. Isolates still able to quench QS after heat treatment mainly belonged to the genera *Arthrobacter*, *Pseudomonas* and *Delftia*.

3.5. Remaining QQ activity after treatment with proteinase K

The results of the experiments with proteinase K are shown in Fig. 4 for isolates yielding significantly ($p \leq 0.05$) higher values for proteinase K treated samples than untreated ones. We can clearly distinguish between isolates that are still able to quench QS after treatment with proteinase K (data not shown), and those whose QQ activity is completely abolished by proteinase K. We hypothesize that the latter isolates use lactonases or acylases for QQ, while non-enzymatic mechanisms are likely to be involved in the former group. A third group of isolates are those whose QQ activity is reduced, but not fully inhibited by proteinase K treatment. A possible explanation for these findings is the production of a combination of active compounds and/or the presence of enzymes that are not degraded by proteinase K.

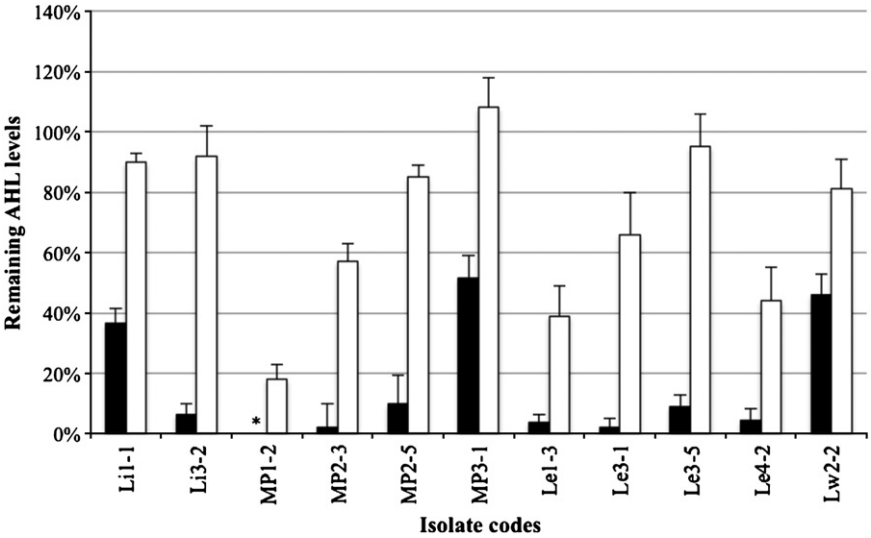

Fig. 4. Remaining AHL levels after proteinase K treatment. Data shown are remaining AHL levels (averages ± SEM) in the sterile supernatant of the isolates after incubation of the AHLs with untreated cell cultures (black bars) and cell cultures treated with proteinase K (white bars) ($n \geq 12$). Isolates for which no remaining AHL levels could be detected are indicated by an asterisk replacing the bar.

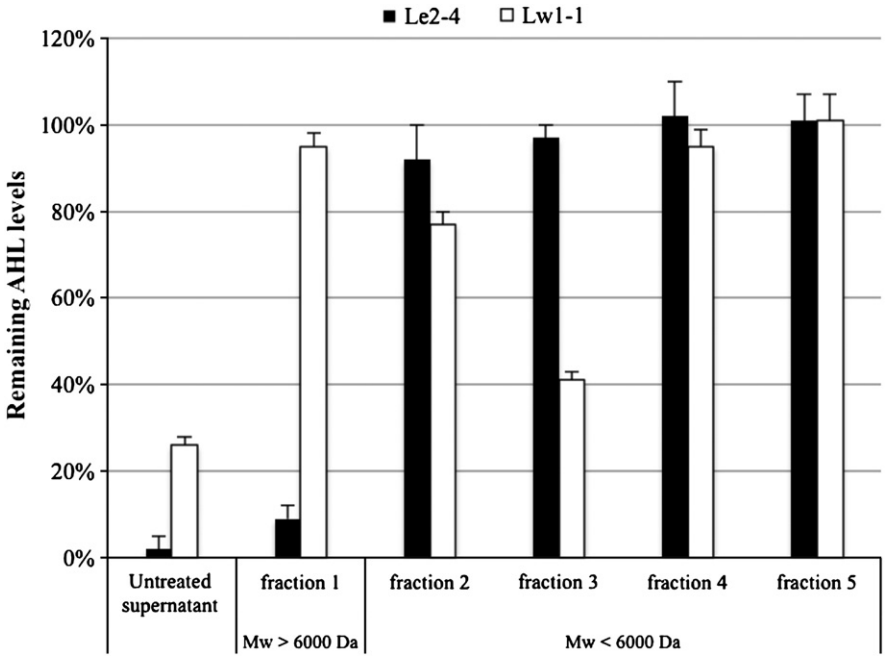

Fig. 5. Remaining AHL levels of untreated supernatant and the different fractions obtained after SEC of the culture supernatant. Data shown are averages ± SEM.

3.6. QQ activity in various fractions obtained with SEC

The remaining AHL levels in the different fractions of supernatant treated with size exclusion chromatography are shown in Fig. 5. For *Delftia* sp. Le1-2 the QQ activity is only present in the fraction containing molecules larger than 6000 Da, indicating that the QQ activity in this isolate is most likely due to enzymatic degradation of the signal molecules. In contrast, for *Diaphorobacter* Lw1-1, no detectable QQ activity was present in the fraction containing the large molecules (>6000 Da). However, two fractions of the supernatant of this isolate containing lower molecular weight compounds did exhibit QQ activity, indicating the secretion of a low molecular weight inhibitory compound (Fig. 5).

4. Conclusions

In the present study we developed and evaluated a high-throughput approach for the isolation of QQ bacteria from environmental samples. We applied this method to 16 samples and isolated a total of 59 strains able to use AHLs as sole sources of C and N, either alone or as part of a consortium. Of these 59 isolates, 41 were able to quench QS in the absence of other partners and 14 possess extracellular QQ activity. Heat treatment did not fully inhibit QQ activity in all isolates, indicating the production of (a) heat-stable QQ molecule(s). Some isolates lose QQ capacity after proteinase K treatment, while others remain able to quench QS. Therefore, it seems plausible that some isolates produce and secrete heat-stable, low molecular weight inhibitory compounds. The results of the SEC experiments further support this hypothesis. Future research will have to reveal the chemical structure of these active molecules as well as their mechanism of action.

Acknowledgements

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