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ORIGINAL PAPER

A novel medium for the isolation of *N*-acylhomoserine lactone-degrading bacteria

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Abstract A novel chemically defined medium, named KG medium, supplemented with *N*-3-oxo-hexanoylhomoserine lactone (3-oxo-C6-HSL), an acylhomoserine lactone (AHL) used as signalling molecules in Gram-negative bacterial cell-to-cell communication, as the sole source of carbon and nitrogen, was designed and successfully used for the enrichment and isolation of AHL-degrading bacteria. A 3-oxo-C6-HSL-degrading bacterium, 13sw7, was isolated from sewage after six enrichment transfers in the 3-oxo-C6-HSL-containing KG medium. On the basis of the almost complete 16S ribosomal DNA sequence, isolate 13sw7 was clustered with unculturable β-proteobacteria. This study indicates that the AHL-containing KG medium is effective in isolating AHL-degrading bacteria, including those previously considered unculturable, from environmental sources. To the best of our knowledge, this is the first documentation of the isolation of an AHL-degrading proteobacterium from sewage.

Keywords KG medium · *N*-3-oxo-hexanoylhomoserine lactone · Proteobacterium · Quorum sensing · Quorum quenching · Sewage

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Introduction

Gram-negative bacteria cell-to-cell communication, known as quorum sensing (QS), is achieved via the production and sensing of signalling molecules called *N*-acylhomoserine lactones (AHLs). LuxI homologues are responsible for the synthesis of AHL molecules, which accumulate in the surrounding environment. At high cell densities, the AHL reaches a threshold concentration at which it interacts with the LuxR homologue, which then regulates transcription of a number of target genes. These include genes that encode biofilm formation, bioluminescence, antibiotic production, and production of extracellular virulence factors [5, 6, 14, 16]. Thus, QS is an interesting target to attenuate bacterial virulence and interruption of QS, known as quorum quenching, can be achieved by directly inactivating the AHL molecules or targeting the receptor protein. Hence, quorum quenching may represent a novel anti-bacterial method to control bacterial infections [15].

Quorum quenching owing to degradation of AHL molecules via enzymatic activities was first reported by Dong et al. [3, 4] in the Gram-positive *Bacillus* species which inactivates AHLs via lactonase activity. Subsequently, acylase from *Pseudomonas aeruginosa* PAO1 has been reported to hydrolyse the *N*-acyl side chain from the lactone ring [10].

Quorum quenching bacteria have been isolated from soil [1] and rhizosphere [12]. Leadbetter and Greenberg [8] have reported the composition of a comprehensive medium for isolating soil bacteria that can metabolise AHL as the sole source of energy and nitrogen. Chan et al. [1] have reported a simple, rapid, and efficient method for isolating quorum quenching bacilli from Malaysian rainforest soil. However, no selection medium was designed in that study.

Herein, we describe a novel chemically defined medium, named KG medium, supplemented with *N*-3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL), for the isolation of AHL-degrading bacteria. We also describe a 3-oxo-C6-HSL-degrading bacterium, 13sw7, isolated from sewage.

Materials and methods

Bacterial strains, growth media, and culture conditions

The bacterial strains used were *Escherichia coli* DH5α, the biosensor *Chromobacterium violaceum* CV026 [9], and isolate 13sw7, purified from a local sewage sample as described below.

All bacteria were routinely cultured in Luria–Bertani (LB) medium (in grams per litre: tryptone, 10; yeast extract, 5; and NaCl, 5), broth or agar, buffered with 50 mM 3-[*N*-morpholino] propanesulfonic acid to pH 6.8 to prevent spontaneous degradation of AHLs. For enrichment and isolation of quorum quenching bacteria, KG medium was used. A basal medium was first prepared and it contained (in grams per litre) NaCl, 1.0; KCl, 0.5; $MgCl_2$ 0.4; $CaCl_2$, 0.1; Na_2SO_4, 0.15; KH_2PO_4, 5.0; and 2-(*N*-morpholino)-ethanesulfonic acid (MES), 1.0. Its pH was adjusted to 5.5 with 1 M NaOH. The basal medium was autoclaved and cooled. Finally, an appropriate volume of a sterile stock solution of trace elements was added aseptically to final concentrations of 1 mg of $FeCl_3$, 0.1 g of $MnCl_2$, and 0.46 g of $ZnCl_2$ per litre of basal medium prepared above. Where necessary, growth substrates were added to sterile KG medium.

For liquid KG medium containing 3-oxo-C6-HSL as the sole source of carbon and nitrogen, a stock solution of 3-oxo-C6-HSL was dispensed into a sterile tube, the acetonitrile was removed by evaporation under a stream of cool air, and sterile KG medium was added to rehydrate the remaining 3-oxo-C6-HSL at a final concentration of 500 μg/ml. The 3-oxo-C6-HSL-containing KG medium was used immediately after preparation.

Where necessary, growth media were supplemented with ampicillin (100 μg/ml). Unless otherwise stated, sewage bacteria and CV026 were grown at 28°C and *E. coli* was grown at 37°C.

Enrichment and isolation of AHL-degrading bacteria from sewage

To isolate AHL-degrading bacteria from sewage, 1 g of sewage sample (obtained from a domestic sewage treatment plant) was resuspended in 10 ml of KG medium and vortexed vigorously for 10 min. Two millilitres of this suspension was briefly spun (7,000×*g*) to remove any particles. The supernatant was centrifuged at 13,000×*g* and the pellet was washed with KG medium and resuspended in 2 ml of KG medium. Following this, 100 μl of the suspension was inoculated into 3 ml of KG medium containing 3-oxo-C6-HSL (500 μg/ml) in an 18-mm-diameter tube and incubated at 28°C with shaking (220 rpm). After 48 h, a 10% (v/v) transfer was made to fresh 3-oxo-C6-HSL-containing KG medium to enrich for 3-oxo-C6-HSL-metabolizing bacteria. Similar transfers to fresh enrichment medium were made after each 48 h of incubation. At the sixth enrichment cycle, a diluted suspension was plated onto LB agar. Single colonies were picked and repeatedly streaked on LB agar to obtain pure colonies.

Preparation of isolate 13sw7 resting cells

Bacterial isolate 13sw7 cells were grown overnight at 28°C with shaking (220 rpm) in LB broth. Cells, 100 ml, were centrifuged (10,000×g) for 10 min at 4°C. The cells were resuspended in 100 ml of PBS (100 mM, pH 6.5) and washed twice with the same buffer. The cells were finally resuspended in 10 ml of PBS (100 mM, pH 6.5) to OD_{600} of 1.0 and used as a source of resting cells for in vitro AHL-inactivation assay.

AHL-inactivation assay

Aliquots of 2 μl of 3-oxo-C6-HSL in acetonitrile were dispensed into 1.5 ml sterile tubes where the solvent was evaporated to dryness. The tubes were then filled with 200 μl of resting 13sw7 cell suspension, rehydrating the 3-oxo-C6-HSL to a final concentration of 0.025 μg/μl. The resting cell suspensions were incubated at 28°C with shaking (220 rpm). Samples of 10 μl were withdrawn at 0, 3, 6, and 9 h.

The reaction was stopped by heating as described previously [1]. After cooling, the reaction mix (10 μl) was spotted onto CV026 lawn, followed by an overnight incubation at 28°C. Control experiments involving extraction buffer (PBS) and *E. coli* DH5α incubated with 3-oxo-C6-HSL were also performed.

Molecular analysis of isolate 13sw7

The 16S ribosomal DNA (rDNA) universal primers 27F [13] and 1525R [2] were used for 16S rDNA PCR with 13sw7 genomic DNA as the template. PCR amplification and purification of 16S rDNA products were carried out as described previously [1]. PCR products were ligated into pGEM-T by using the pGEM-T Vector System (Promega, USA). DNA sequencing was performed by routine automated methods in which standard M13 forward and reverse primers and primers previously designed to anneal to internal

target regions of the 16S rDNA of most bacteria were used [7]. The nucleotide sequence of a PCR-amplified fragment of the 16S rDNA of isolate 13sw7 was determined as described previously [1] and we used MEGA version 4 [11] to perform phylogenetic and molecular evolutionary relationship analyses. A phylogenetic tree was generated by using the neighbour-joining algorithm and rooted with an appropriate outgroup to show the direction of evolution. Bootstrap analyses for 1,000 re-samplings were performed to provide confident estimates for tree topology.

Other analyses

Gram determination was performed using a kit from Roche (USA) and following the manufacturer's instructions. Cell morphology was observed with a Nikon (Japan) light microscope at ×400 and ×1,000 magnifications.

Nucleotide sequence accession number

The 16S rDNA sequence of isolate 13sw7 was assigned GenBank accession no. FJ179171. All other rDNA sequences were from GenBank.

Results and discussion

We have designed a chemically defined selection medium, named KG medium, supplemented with 3-oxo-C6-HSL, for the enrichment and isolation of AHL-degrading bacteria. KG medium is inexpensive and easy to prepare.

KG medium containing 3-oxo-C6-HSL was inoculated with sewage sample and the medium turned turbid within 48 h, indicating the occurrence of bacterial growth during the enrichment process. No growth was evident in the control KG medium without 3-oxo-C6-HSL (data not shown). Pure colonies were obtained by repeated streaking on LB agar. A number of bacterial isolates with different morphologies were observed and isolate 13sw7 was selected for further analysis.

Isolate 13sw7 was a slow growing aerobic bacterium and its colonies were only visible after incubation at 28°C for 72 h. On LB medium, isolate 13sw7 formed small, pinpoint, yellowish colonies. Cells from LB broth culture were small coccobacilli as observed under a light microscope. All experiments reported here were performed at 28°C, although cultures also grew at 37°C.

Two findings demonstrated the successful isolation of AHL-degrading or quorum quenching bacteria from sewage. Firstly, AHL-metabolizing bacteria were selected through the use of repeated cycles of enrichment process and fresh KG medium containing 3-oxo-C6-HSL as the sole source of carbon and nitrogen. Secondly, isolate 13sw7 did not produce any chemical compound that inhibited CV026 or any compound that altered the pH of KG medium from acidic to basic, leading to breakdown of 3-oxo-C6-HSL.

To verify isolate 13sw7 did not produce any chemical compound that interfered with the detection of AHLs by the biosensor CV026, we extracted bacterial culture supernatants twice with ethyl acetate. The extract was analysed by reverse thin layer chromatography. Isolate 13sw7 did not produce any detectable active compound that inhibited the detection of 3-oxo-C6-HSL by CV026 (data not shown) or inhibited the growth of biosensor CV026. We also examined AHL production by isolate 13sw7. When it was cross-streaked with CV026, no pigment formation was observed, indicating no short-chain AHLs production by this isolate. However, because CV026 is unable to detect long chain AHLs [9], unequivocal confirmation of AHL production requires mass spectrometry analysis.

The ability of isolate 13sw7 resting cells to inactivate 3-oxo-C6-HSL was confirmed by the AHL-inactivation assay. The resting cells of isolate 13sw7 degraded 3-oxo-C6-HSL molecules in the medium within 9 h (Fig. 1), suggesting a rapid turnover of 3-oxo-C6-HSL molecules under the experimental condition. No degradation of 3-oxo-C6-HSL molecules was obvious when the AHL-inactivation assays were repeated with *E. coli* DH5α cells.

AHL molecules are known to undergo lactonolysis in basic medium and at high temperatures [17]. We verified that 3-oxo-C6-HSL did not degrade in KG medium, which was buffered with MES. The pH of the 3-oxo-C6-HSL-containing KG medium, inoculated with bacteria, was measured at each

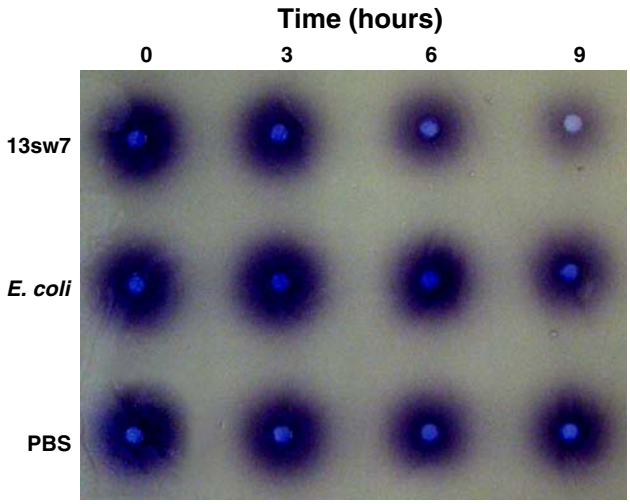

Fig. 1 Detection of 3-oxo-C6-HSL-degrading isolate 13sw7. Reaction mixtures were spotted onto biosensor CV026 lawn as described in "Materials and methods". Disappearance of 3-oxo-C6-HSL was evident by decreased or loss of pigment formation on CV026 lawn. Rows from *top to bottom*: 13sw7 (isolate 13sw7), *E. coli* (*E. coli* DH5α), and PBS (PBS buffer, 100 mM, pH 6.5)

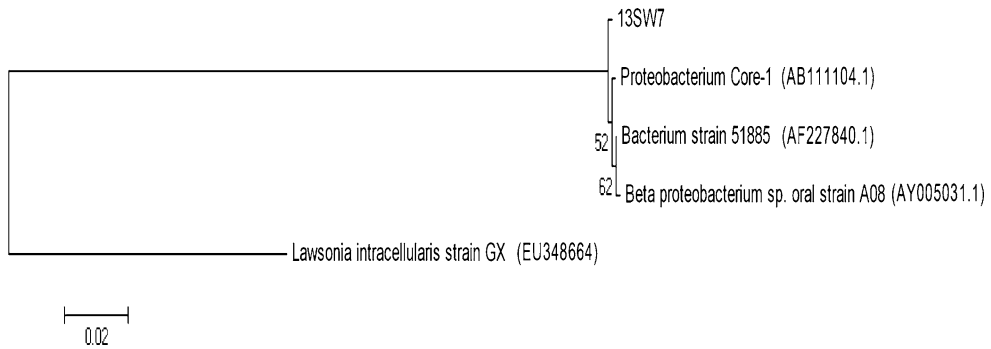

Fig. 2 16S rDNA-based phylogenetic tree showing the phylogenetic position of isolate 13sw7. A total of 1,361 unambiguously aligned nucleotides were used in a 1,000-step *MEGA* 4 analysis. The *bar* represents evolutionary distance as 0.02 changes per nucleotide position, determined by measuring the lengths of the *horizontal lines* connecting the species. The *numbers* (bootstrap values as percentages of 1,000 replications) provide support for the robustness of the adjacent nodes. *L. intracellularis* served as outgroup. *Numbers in parenthesis* are GenBank accession numbers

sampling point and compared to that of the non-inoculated control 3-oxo-C6-HSL-containing KG medium. No significant change of pH was observed during the enrichment process (data not shown). Furthermore, 3-oxo-C6-HSL remained stable under the experimental condition for at least 96 h when incubated at 28°C (data not shown).

An almost complete sequence, comprising 1,361 nucleotides, of the 16S rDNA of isolate 13sw7 was determined and analysed. Web-based similarity searches against the GenBank database revealed that the 16S rDNA of 13sw7 shared 99% sequence similarity with the 16S rDNA of a poorly characterised proteobacterium (Genbank accession no. AB111104.1) and 13sw7 was phylogenetically clustered with unculturable proteobacterium phylum (Fig. 2). This is the first documentation of the use of the novel KG medium for selecting from sewage a quorum quenching bacterium that clustered closely to previously considered as unculturable β-proteobacterium.

Leadbetter and Greenberg [8] have reported the use of a complex defined medium, comprising comprehensive vitamins, trace elements, and selenate-tungstate, incorporated with 3-oxo-C6-HSL as the sole source of energy and nitrogen to isolate *Variovorax paradoxus* capable of degrading AHLs. In comparison, KG medium mainly consists of basal medium and trace elements. When supplemented with 3-oxo-C6-HSL, KG medium was effective in isolating AHL-degrading bacteria from sewage. We have also successfully used KG medium supplemented with *N*-(3-oxotetradecanoyl)-L-homoserine lactone (3-oxo-C14-HSL, a long chain AHL) to isolate novel AHL-degrading bacteria from soil, faeces, and rhizosphere (unpublished data). Hence, we believe that this inexpensive and easily prepared KG medium will enable us to isolate more novel AHL-degrading and -inactivating bacteria from different environmental sources in our quest to identify effective quorum quenching bacteria and their AHL-hydrolysing enzymes as agents of biocontrol.

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