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A Novel Bioassay for High-Throughput Screening Microorganisms with *N*-acyl Homoserine Lactone Degrading Activity

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Abstract A novel biosensor strain (*Escherichia coli* ALM403) that responded to *N*-acyl homoserine lactone (AHL) was constructed using a *luxR-Plux* cassette as a regulatory sequence and β-mannanase as a reporter gene. Dinitrosalicylic acid method was used to detect the response of the sensor strain to *N*-acyl homoserine lactone. By investigating the response to a range of concentrations of *N*-β-oxooctanoyl-L-homoserine lactone (OOHL), it was demonstrated that the expression of mannanase in *E. coli* ALM403 could be greatly enhanced by OOHL and resulted in an assayable phenotype. A high-throughput screening approach was developed to isolate AHL-degrading microorganisms, and a marine *Halomonas* sp. S66-4 showing a marked AHL-degrading ability was successfully isolated. In conclusion, the bioassay system provided a simple and efficient approach to isolate AHL-degrading bacteria.

Keywords *N*-Acyl homoserine lactone · Quorum sensing · Quorum quenching · Bioassay · AHL-degrading bacterium

Introduction

Numerous bacteria monitor their own population densities by sensing the concentration of small signal molecules, which further regulate the expression of specific genes in response to novel environmental challenges. This phenomenon is termed quorum sensing [1]. In many

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bacteria, signal molecules belong to a family of (*N*-acyl homoserine lactones) AHLs. Quorum sensing is involved in the regulation of various bacterial behaviors, such as bioluminescence, Ti plasmid conjugal transfer, and swarming motility. In particular, quorum sensing regulates the production of exoenzymes and virulence proteins in several pathogens, such as *Pseudomonas aeruginosa*, *Erwinia carotovora*, and *Burkholderia cepacia*, and further influences their pathogenic abilities [2]. This mechanism suggests that it might be possible to control bacterial disease by interfering with the quorum-sensing system; this is termed quorum quenching [3]. Several AHL-degrading enzymes, including AHL lactonases and AHL acylases, have been isolated from different microorganisms and have been shown to attenuate pathogenicity [4–6].

Bioassays with sensor strains are widely employed to detect AHLs due to their practicality and high sensitivity [7–10]. Several detectable phenotypes, including the activity of β-galactosidase encoded by the *lacZ* gene [7], bioluminescence encoded by the *luxCDABE* locus [8], green fluorescence encoded by the *gfp* gene [9], and the production of violacein pigment [10], have been used to reveal the responses of sensor strains to exogenous AHL molecules. Different methods based on sensor strains have been adopted for detecting AHLs and screening AHL-degrading microorganisms [11]. For example, thin-layer chromatography (TLC) is an efficient method to separate and detect AHL molecules [12]. However, because of low plate usage (in general, one plate can only monitor one sample), the TLC method is only limited to check whether AHLs are produced and is unsuitable for isolating bacteria producing or degrading AHLs from a large number of samples. To detect multiple samples with AHL-degrading activity simultaneously, various methods based on different biosensors and reporter genes were developed [4, 13, 14]. Dong et al. developed *lacZ*-based agar slice method and found the first lactonase successfully [4]. Seo et al. described a diffusion halo method based on sensor strain *Chromobacterium violaceum* CV026 and isolated an AHL-degrading bacterium [13]. Mei et al. obtained 37 strains that exhibited AHL inactivated activity from 2,000 isolates by a similar method using sensor strain *Agrobacterium tumefaciens* strain NTL4(pZLR4) [14]. These methods could be used for isolating AHL-degrading microorganisms, but were unsuitable for microplate assay and high-throughput screening. For the rapid screening of AHL-degrading bacteria, Jafra and colleagues developed a microtiter assay method using *gfp*-based sensor strain [15]. But detection of green fluorescence is very difficult by naked eyes and generally requires a fluorometer or epifluorescence microscope.

In this study, we constructed a novel sensor strain using the *lux* regulator elements derived from *Vibrio fischeri* and a β-mannanase gene (*man*) from *Bacillus subtilis* as a reporter gene. Endo-β-1,4-mannanase (EC.3.2.1.78) catalyzes the random hydrolysis of β-1,4-mannopyranosyl linkages within the mannan backbone, releasing short- and long-chain oligomannosides [16]. The mannanase activity could be conveniently determined by dinitrosalicylic acid (DNS) method [17]. Using the bioassay, an AHL-degrading bacterium was successfully isolated.

Materials and Methods

Chemicals

N-β-Oxooctanoyl-L-homoserine lactone (OOHL) was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Konjac flour was purchased from Wuhan Qingjiang Konjac Co., Ltd. (Wuhan, China). DNS reagent was prepared as described by Miller [17].

Bacterial Strains and Culture Conditions

Escherichia coli strain DH5α was used as a host for DNA cloning. *B. subtilis* strain BME0437, which provided the *man* gene, was isolated from Shizi Mountain in Wuhan, China, and was deposited in the China Center for Type Culture Collection (CCTCC no. AB209228). *A. tumefaciens* strain NT1 (*traR*; *tra*::*lacZ749*) was kindly provided by Dr. Lianhui Zhang and served as an AHL sensor strain. Some marine bacteria from the Marine Culture Collection of China (MCCC) were detected for their AHL-degrading abilities.

E. coli strain DH5α was cultured in Luria–Bertani (LB) medium at 37 °C. *B. subtilis* strain BME0437 was cultured in LB at 28 °C. Marine bacteria were cultured in modified LB medium (1 % tryptone, 3 % NaCl, 0.5 % yeast extract, pH 7.0) at 28 °C. Liquid mannan (MAN) medium containing 0.1 % konjac flour, 0.4 % yeast extract, 0.02 % $MgCl_2$, and 0.1 % KH_2PO_4 served as AHL bioassay medium for the DNS method. *A. tumefaciens* strain NT1 (*traR*; *tra*::*lacZ749*) was cultured in a minimal medium, as described by Zhang [18]. Antibiotics were added when appropriate at the following concentrations: ampicillin, 100 μg ml^{-1}, and chloramphenicol, 25 μg ml^{-1}.

Biosensor Strain Construction

The *man* gene (GenBank accession number GQ988368) was amplified by PCR using the primers F-*man*-*Sph*I: GCGGCATGCTTAAGAAACATACGATCT and R-*man*-*Hin*dIII: TATAAGCTTATTCCGCGATCGGCGTCA, from the genomic DNA of *B. subtilis* strain BME0437. The PCR product was cloned into plasmid pUC19 to generate plasmid pUC-*man*. Then, the *lux* cassette, containing the *luxR* gene, *lux* promoter (P*lux*), and ribosome binding site (RBS), was obtained by PCR from plasmid pSB403 (a kind gift from Paul Williams) using primers F-*lux*403-*Bam*HI: GACGGATCCTTAATTTT TAAAGTATGGG-3 and R-*lux*403-RBS-*Sph*I: ACAGCATGCTCATAGTTAATTTCTC CTCTTTAATGGTACCTACGTAACCAACCTCCCTT-3. The purified PCR product was first digested with *Bam*HI for 4 h, followed by partial digestion with *Sph*I (due to the *Sph*I site contained in the *lux* cassette). Fragments of 1,018 bp were recovered and inserted into pUC-*man* to form pULM403. Plasmid pULM403 was digested with *Bam*HI and partially digested with *Hin*dIII to generate a 2,123-bp fragment containing the *luxR*-P*lux*-*man* cassette, which was recovered and cloned into plasmid pACYC184 to obtain pALM403. The process of plasmid construction is shown in Fig. 1. Plasmid pALM403 was transformed into *E. coli* strain DH5α to generate the sensor strain *E. coli* ALM403.

DNS Method to Detect AHLs

E. coli strain ALM403 was cultured in LB medium at 37 °C overnight and diluted five times in liquid MAN medium supplemented with chloramphenicol and test samples. After incubation for an appropriate time period at 37 °C, the bacterial culture (100 μl) was mixed with dinitrosalicylic acid reagent (100 μl) and heated in a 95 °C water bath for 10 min. The visible color change of the reaction mixture could be directly observed to identify the presence or absence of AHL molecules. Moreover, the mannanase activity could accurately be determined by measuring the absorbance at 540 nm with a Multiskan Spectrum (Thermo Scientific, USA).

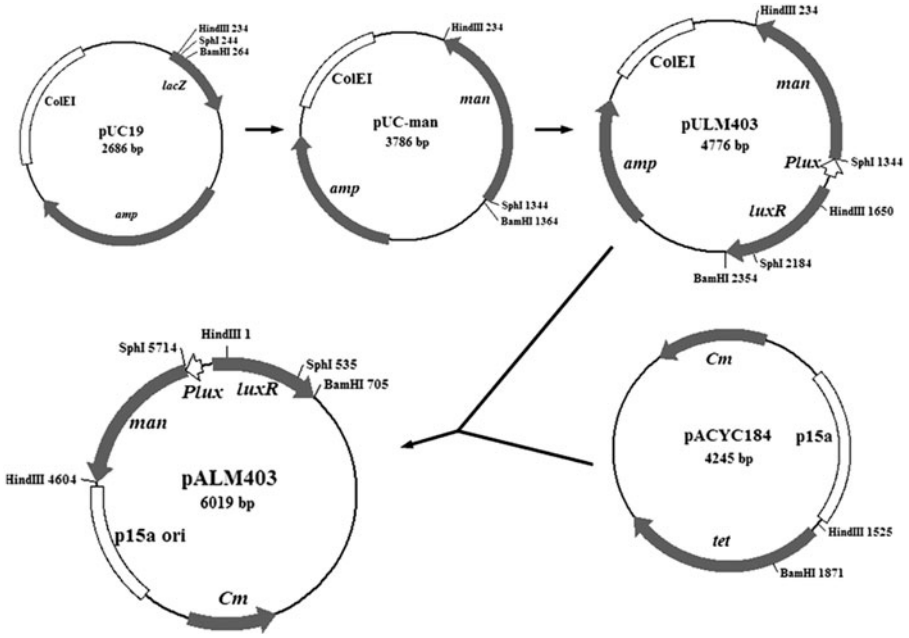

Fig. 1 Construction of plasmid pALM403. The *man* gene was inserted into pUC19 to form pUC-*man*. The *lux* cassette containing the *luxR* gene and *Plux* promoter was cloned into pUC-*man* to generate pULM403. The DNA fragment containing the *lux* cassette and the *man* gene was separated from pULM403 by restriction and ligated into pACYC184 to obtain plasmid pALM403

Agar Slice Method to Screen AHL-Degrading Bacteria

The solid minimal medium was supplemented with 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-Gal) to a final concentration of 40 μg/ml and cut into separated slices. Cultivated test isolates were reacted with AHLs for a certain time, and then the reaction mixtures were dotted on one end of the agar slices. Later the cultures of sensor strain *A. tumefaciens* NT1 (*traR*; *tra*::*lacZ749*) were spotted at progressively further distances from the location of the tested sample, and after incubation for 24 h, the maximum distance from the last blue sensor strain to the origin of the tested sample was calculated to represent the amount of residual AHLs.

Isolation of AHL-Degrading Bacteria

Marine bacterial isolates were cultured at 28 °C with gentle shaking. When the OD_{600} reached about 1.5, 50 μl of bacterial suspension was mixed with an equal volume of 10 μmol l^{-1} OOHL in a 96-well plate. After incubation at 28 °C for 4 h, the mixtures were heated in a 95 °C water bath for 5 min to inactivate any mannanases produced by the strains. Then, 15 μl of each reaction mixture, 30 μl of the overnight culture of *E. coli* strain ALM403, and 105 μl of liquid MAN medium were pipetted to a 96-well plate and incubated for 4 h at 37 °C. Then, 70 μl of each culture and 70 μl of dinitrosalicylic acid reagent were transferred to a 96-well plate and incubated at 95 °C for 10 min. The test strains that resulted in a light color were chosen for further determination.

Candidate strains were re-cultured and reacted with 10 μmol l^{-1} of OOHL as described above. The reaction mixtures were further assayed by the DNS method described above and agar slice method involving *A. tumefaciens* strain NT1 (*traR*; *tra*::*lacZ749*) [4].

Results

Principal of Response to AHLs of *E. coli* ALM403

Plasmid pALM403 was constructed by combining the *luxR-Plux* cassette, which contains the *luxR* gene and the *lux* promoter derived from *V. fischeri*, with the *man* gene to achieve inducible expression of the mannanase. In *E. coli* strain ALM403, mannanase was synthesized at a low level in the absence of AHLs. In the presence of exogenous AHLs, the expression of the mannanase was greatly enhanced, and more β-mannanase was produced, resulting in an assayable phenotype.

Principal and Effectiveness of DNS Method

DNS reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid, which absorbs light strongly at 540 nm. The level of absorbance varied depending on the amount of reducing sugar, which was determined by the amount of mannanase present [17]. Based on this principle, we developed a DNS bioassay to detect AHL using *E. coli* strain ALM403.

To investigate the response of the sensor strain to OOHL using the DNS method, *E. coli* strain ALM403 was incubated with 1 μmol l^{-1} of OOHL as described above. Samples from the cultures were withdrawn every 1 h and reacted with DNS. One hundred microliters of the reaction mixtures was added to a microtiter plate to observe the color change directly (Fig. 2a) or to measure the absorbance at 540 nm (Fig. 2b). As expected, the color difference in the presence or absence of OOHL could be distinguished after a 4-h incubation period. Our results indicated that expression of mannanase increased little in the absence of OOHL, but was rapidly induced in the presence of 1 μmol l^{-1} of OOHL.

To determine the response of the sensor strain to different concentrations of OOHL, an overnight culture of *E. coli* strain ALM403 was diluted five times in liquid MAN medium. OOHL was added at a range of concentrations, and the cultures were further incubated for 4 h at 37 °C and reacted with DNS reagent. One hundred microliters of the reaction mixtures was added to a microtiter plate for direct observation (Fig. 2c) or absorbance measurement at 540 nm (Fig. 2d). As shown in Fig. 2c and d, in the presence of 50 nmol l^{-1} of OOHL, the increased expression of mannanase resulted in an obvious visible variation in phenotype (Fig. 2c). When the concentration of OOHL reached or exceeded 0.5 μmol l^{-1}, the maximum activity of mannanase was obtained, which was about 20-fold higher than that in the absence of OOHL. This result demonstrated that the expression of mannanase in *E. coli* strain ALM403 varied according to the concentration of OOHL, though the expressions were not linear with respect to the concentration of OOHL.

Screen of AHL-Degrading Microorganisms

To investigate the effectiveness of *E. coli* strain ALM403 for screening AHL-degrading bacteria, we assayed the AHL-degrading ability of marine bacteria supplied by the MCCC. One isolated strain, *Halomonas* sp. S66-4 (CCTCC no. AB209229 and GenBank accession

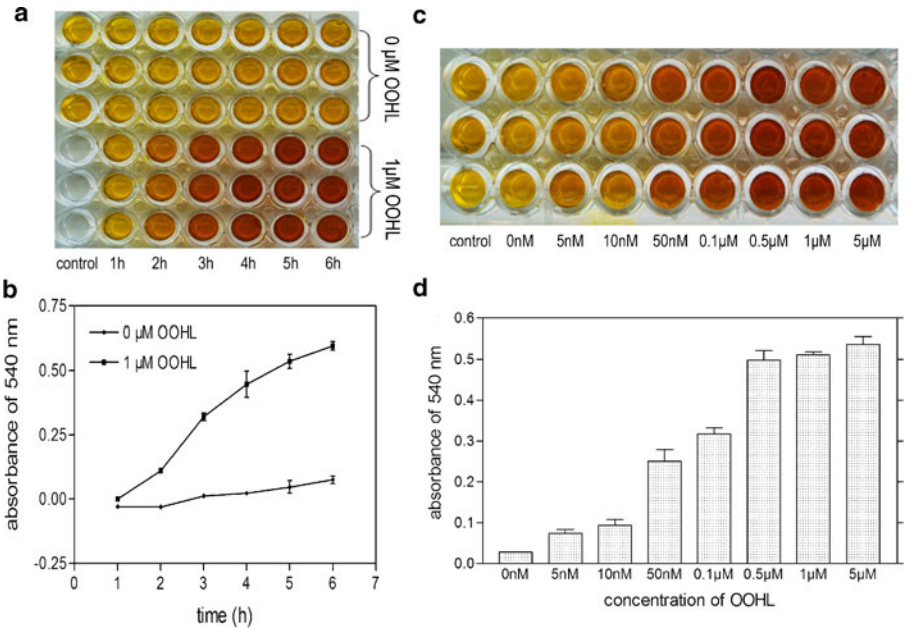

Fig. 2 Response of *E. coli* strain ALM403 to OOHL, as determined by the DNS method, **a** and **b** *E. coli* strain ALM403 response time to OOHL. **a** Direct observation and **b** absorbance measurement at 540 nm. **c** and **d** *E. coli* strain ALM403 response sensitivity to different concentrations of OOHL. OOHL final concentrations: 0, 5, 10, and 50 nmol l^{-1}; 0.1, 0.5, 1, and 5 μmol l^{-1}. Mannanase activity was measured according to the DNS method. **c** Visible color distinction of reaction mixtures. **d** Bar diagram of the absorbance values. Each experiment was performed in triplicate, and *error bars* denote the standard deviation from the mean

number of the 16S rDNA sequence: EU670604), showed strong degradative activity against OOHL. Further assays using the DNS method and agar slice method were performed to determine the AHL-degrading activity of *Halomonas* sp. strain S66-4. As shown in Fig. 3, the results of both of the methods confirmed that 10 μmol l^{-1} of OOHL was completely

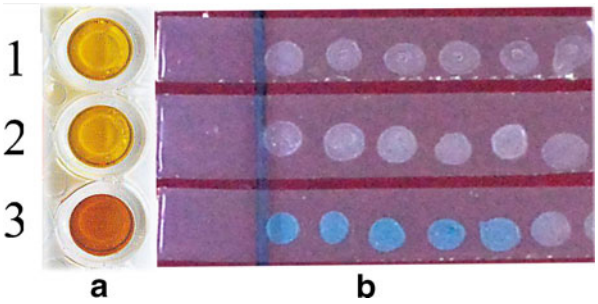

Fig. 3 Detection of the AHL-degrading activity of *Halomonas* sp. strain S66-4 using two different bioassays. *Row 1* negative control: PBS buffer free of OOHL. *Row 2* reaction product of 50 μl of *Halomonas* sp. strain S66-4 culture with 50 μl of 10 μmol l^{-1} OOHL at 28 °C for 4 h. *Row 3* positive control: reaction product of 50 μl of PBS buffer with 50 μl of 10 μmol l^{-1} OOHL at 28 °C for 4 h. **a** DNS method. **b** Agar slice method: 5 μl of test samples was added to one side of an agar slice supplemented with X-Gal. Then a culture of the sensor strain, *A. tumefaciens* strain NT1 (*traR*; *tra*::*lacZ749*), was spotted sequentially onto the agar slice and cultivated at 28 °C for 24 h

degraded by *Halomonas* sp. strain S66-4 within 4 h. These results demonstrated that *E. coli* strain ALM403 could be used to screen AHL-degrading bacteria by the DNS method.

Discussion

To assay the activity of mannanase, we adopted the MAN medium containing konjac flour composed of glucose and mannose subunits with β-1, 4 linkages. In liquid MAN medium, 0.1 % konjac flour was used as higher concentrations could not be fully dissolved. We also investigated the applicability of another substrate of mannanase, locust bean gum (LBG), which mainly consisted of galactomannan, composed of galactose and mannose units. Results were similar to those with konjac flour (data not shown). However, compared with konjac flour, LBG was of lower solubility, and it was difficult to obtain even distribution throughout the medium. Therefore, we recommended konjac flour as the mannanase substrate in the DNS method.

Different quorum-sensing bacteria produce different AHL molecules, which share a conserved homoserine lactone ring, and differ in the length of their side chains (C4 to C12) and in the levels of substitution and saturation at carbon 3. Correspondingly, different R-type receptor proteins are synthesized to recognize their cognate AHL molecules. Receptor proteins from different bacteria possess strong or weak affinity to heterologous AHL molecules and present different responding abilities. Cha et al. [19] investigated four sensor strains, based on *tra* of *A. tumefaciens*, *lux* of *V. fischeri*, *las* of *P. aeruginosa*, and pigment production by *C. violaceum*, for their capacity and sensitivity to detect sets of different AHL molecules. They found that the *tra* biosensor strain detected the broadest range of AHL derivatives and showed the best sensitivity to a microdose of AHLs. Compared with the *tra* biosensor, the sensor strain based on *lux* cassette could detect most of the 3-oxo- and alkanoyl AHL derivatives, but showed lower intensity and sensitivity to the same microdose of AHL molecules [19]. In addition, Andersen et al. [9] developed a sensor strain using a *luxR-Plux*-RBSII cassette and the *gfp* gene, and investigated its response to different AHL molecules. Their results revealed that OHHL, which was the cognate signal molecule of *luxR-Plux* cassette, induced the maximum amount of *gfp* expression in the sensor strain, whereas OdDHL showed almost no induction, and other AHL molecules displayed approximately tenfold less induction. The biosensor strain (*E. coli* ALM403) adopted the same regulatory element as Andersen's [9]. Referring to the results above, our sensor strain (*E. coli* ALM403) was expected to possess the same response specificity to different AHL molecules. Hence, known types and concentrations of AHLs, for example OOHL, were adopted in the constructing platform for screening bacteria being capable of degrading AHLs. Although *E. coli* ALM403 is not as sensitive and versatile as the *tra* system of *A. tumefaciens* and is unsuitable for screening bacteria producing unknown AHL molecules, this sensor strain showed remarkable and assayable trait variation in the absence or presence of known AHL molecules and was competent enough for screening AHL-degrading microorganisms.

In reality, all of the three methods (agar slice method, diffusion halo method, and DNS method) are able to evidence AHL molecules distinctly through color development and detect multiple samples simultaneously. The agar slice method needs the cutting of solid medium into uniform separated slices, which was tedious and time consuming. The diffusion halo method relied on too much plate area and was of low efficiency in screening. Furthermore, sensor strain *A. tumefaciens* strain NT1 and *C. violaceum* CV026 must be incubated for over 24 h to show color change, which was time consuming. However,the

DNS method can be carried out by micropipet and microplate, and detection based on *E. coli* ALM403 could be finished within 4 h. Hence, it is believed that the DNS method is more efficient, time saving, and suitable for high-throughput screening. Moreover, in comparison with the green fluorescent protein (GFP)-based bioassay method, the DNS method adopted in our study does not rely on special instruments like epifluorescence microscopes or fluorometers to distinguish a changed appearance in the bioassay strain, which is generally easier and might be much more suitable for most of the labs, especially those with no GFP detector.

In this study, we constructed a biosensor strain and established bioassays to detect AHL molecules. Based on the DNS method, we developed a high-throughput screening method for AHL-degrading microorganisms and isolated a marine bacterium *Halomonas* sp. that showed a strong AHL-degrading capacity. Our results demonstrate the effectiveness of this novel bioassay for the detection of AHLs and the screening of AHL-degrading bacteria.

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